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A HISTOLOGICAL STUDY OF THE PARATHYROIDS IN THE ANURA

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FOUR PLATES (TWENTY FIGURES)

AUTHOR'S ABSTRACT

The parenchymal cells in the parathyroids of Rana catesbeiana (Shaw) are found to undergo a very curious cyclic change. The cytoplasm is broken down by a process of cytolysis. The nuclei are decomposed by chromatolysis and pycnosis The entire parenchyma is finally liquefied, except for a layer of cells lining the capsule

The parathyroid body is reconstructed by young cells which are produced from rather definite growth centers The process of regeneration begins some time before cytolysis is completed and continues until the entire organ is restored.

The cytolytic and regenerative processes transpire in such sequence that the amount of apparently healthy tissue present at any one time is a rather large portion of the total mass normally present

The relationship between the parenchymal tissue and the stroma of the parathyroid is described.

CONTENTS

Introduction . 1
Historical 3
Material and methods . 5
Observations and discussion . 5
  The capsule 5
  The parenchyma . . 7
  Blood vessels . 11
  Accessory tissue 15
  Cyclic changes in the parathyroid . . 16
Conclusions and summary . . 32
Bibliography . . . 34

INTRODUCTION

In 1891, Gley described the parathyroids as discrete organs differing from the thyroids, of which they had formerly been considered a part. Tetany and other physiological disturbances were soon attributed to the removal of these minute bodies, and it was found that they could not be removed from certain animals without resulting in their death. These findings stimulated a histological and cytological study of these curious little bodies in a search for some cellular evidence of their functioning. Human tissue and the parathyroids from a rather wide range of mammals have been studied and different cellular phenomena have been emphasized by various workers as the probable index of activity, and a number of cell types have been described. As yet, no satisfactory cytological evidence of their secretory activity has been found. It is the scope of this paper to describe the parathyroids in the frog (particular reference to R. catesbeiana), present the results of certain histochemical tests, and to describe a very unusual cytolytic and regenerative cycle which was found to occur in the parathyroids of Rana catesbeiana.

The histological study of the frog's parathyroids had been quite completely neglected until Romeis ('26) published an interesting paper on the morphology of these bodies in the Anura. It has since been found that parathyroidectomy on R. catesbeiana is followed by a rather characteristic set of symptoms which terminate in the death of the animal (Waggener). These results suggest very strongly that the parathyroids function in the frog in much the same way that they do in the higher forms including the mammals, and should give additional weight to some of the histological findings which are to be presented in this paper. This is especially true when we consider their bearing upon the controversies which have arisen from various attempts to find cytological evidence of parathyroid activity in the higher forms. It is in this connection that the amphibians offer some advantages which promise to be both helpful and significant. They possess parathyroids which are quite simple in structure and which probably rather closely approximate the primitive type in which these organs first put in their appearance phylogenetically. This enables one, by the use of frog material, to avoid some of the complexity of structure which has evolved with the development of the higher forms and at the same time to deal with the functional components of the organ.

The very peculiar cytological changes of a cyclic nature, which were discovered in the parathyroids of R. catesbeiana, are found to occur without a loss of the vital significance of the organ, as was indicated by extirpation experiments (Waggener). The cause and the ultimate significance of these seasonal disturbances are not known; but the time when they occur, together with other concurrent phenomena in the life of the animal, suggests some very alluring possibilities which will be of interest to the physiologist and the zoölogist as well as to the histologist.[1]

HISTORICAL

The parathyroids were discovered in the frog about the same time by Ecker and Leydig, although the latter (Leydig, '52) was the first to give a written account of them. Ecker ('53) thought that these little bodies represented thymus tissue, while Leydig regarded them along with the ventraler Kiemenrest as thyroid tissue. Maurer ('88) was the first to work out the origin of the parathyroids (Epithelkörperchen) in the frog and found that they were separate and discrete bodies, having no connection with thymus or thyroid. In Hertwig's "Handbuch der Entwickelungslehre der Wirbeltiere" (S. 148) Maurer describes them as of characteristic structure unlike either thymus or thyroid. They possess a delicate connective capsule which penetrates between the solid cell cords. They are present throughout life, being demonstrable even in old animals. Krause ('23) also described them briefly, as did Maurer, as consisting of cell cords separated by vascular connective tissue. Romeis ('26, S. 555, 577) has recently given a more complete description. He found that the epithelial bodies (parathyroids) of the Anura are throughout life purely epithelial (epithelioid) structures, whose interior is completely free from connective tissue and blood vessels. The central mass, composed of tortuous cell cords of purely epithelial tissue, is surrounded by a thin, closely woven connective-tissue capsule and a close-meshed subcapsular capillary network.

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[1] I wish to gratefully acknowledge my indebtedness to Prof. B. F. Kingsbury for suggestions, and to thank him for his kind supervision of this work, which was carried out in the Laboratory of Histology and Embryology, Cornell University, Ithaca, New York. A rather more comprehensive discussion of the theoretical aspect of this subject, together with a more lengthy and detailed presentation of facts, may be found in "An experimental and histological study of the parathyroids in the Anura," a thesis presented to the faculty of Cornell University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. A copy of the thesis is on file in the Cornell University Library.

The conflicting findings of Maurer, Krause, and Romeis have to do with a problem of both morphological and physiological significance—a problem which involves the fundamental relationship between the mesenchymal structures and the growing parenchyma of the organ, and, likewise, the extent to which an organ may develop before special provisions for a more intimate contact with the circulating fluids of the body are necessary. With these considerations in mind, parathyroid tissue from R. catesbeiana was examined in order to find out whether or not there is uniformity in the different species of Anura and, at the same time, to try to discover something of the underlying principles which determine this relationship between parenchyma and stroma. This species proved to be a very fortunate choice, since it not only presents many anatomical features which differ widely from those described for other species of Anura, but also offers some very helpful hints for the analysis of the problem suggested above.

Romeis ('26) reported a cyclic change in the microscopic structure of the parathyroids in frogs and toads. He is the first to describe such changes in this organ, although somewhat similar cyclical changes have been recorded for other organs in certain of the Anura. Sklower ('25) described structural changes in the thyroid, thymus, and hypophysis. Braunmühl ('25) has given an account of structural changes occurring in the jugular body. Romeis briefly summarizes his observations as follows:

> Für gewöhnlich zeigen die Epithelkörper einen dicht geschlossenen, ungemein zellreichen Aufbau. Von Zeit zu Zeit erfährt derselbe tiefgreifende Veränderungen: unter zunehmender Vakuolisierung und lebhafter Karyolyse gewinnt das Organ das Aussehen eines epithelialen Reticulums, dessen Maschenräume mit einer nichtfettartigen, eiweissarmen Flüssigkeit gefüllt sind. Von dieser retikulären

Phase lässt sich Schritt für Schritt die unter erneutem Einsetzen von Kernteilungen einsetzende Rückkehr zum geschlossenen, vakuolenfreien Bautypus verfolgen. Die Vermehrung der Parenchymzellen erfolgt für gewöhnlich auf amitotischem Wege. Bei der Rückkehr zum geschlossenen Bautypus ist stets auch das Auftreten mitotischer Kernteilungen festzustellen.

Die Umwandlung zur retikulären Phase, an der sich keinerlei fremde Zellelemente des Mesenchyma beteiligen, ist zwar nicht immer, aber doch am häufigsten Ende Februar und Anfang März zu beobachten, während in den übrigen Monaten der geschlossene Typus vorherrscht. Die Veränderungen werden daher im Sinne eines jahreszeitlich bedingten Zyklus gedeutet (S. 577).

MATERIAL AND METHODS

Tissues from the American bullfrog, Rana catesbeiana (Shaw), were largely used for this study. The animals were procured from the Southern Biological Supply Company in one-dozen lots and were kept in cages and in aquaria for ten days to two months before the tissues were taken. The parathyroids from a small number of R. pipiens and R. palustris were also studied.

Different methods of tissue fixation were employed, which included: Zenker's fluid, Bouin's fluid, Zenker-formol (Helly's fluid), Carnoy's fluid (sublimate mixture), Flemming's fluid, Altmann's fluid, and Benda's fixation method. Fresh material and frozen sections were also studied. General and selective stains were employed, which included: Mayer's haemalum and eosin, Heidenhain's iron hematoxylin, Mallory's connective-tissue stain, Weigert's resorcin fuchsin, sudan III, mucicarmine, brazilin, and Bensley's acid fuchsin and methyl green.[2]

OBSERVATIONS AND DISCUSSION

The capsule

The parathyroid glands in Rana catesbeiana are prevailingly oval to spherical in shape and vary greatly in size, both among individuals and in the same individual. It was frequently observed in this species that a pair of large parathyroids (one on either side) would be accompanied by a pair of small ones which appeared to be as much below the average size as the larger pair was above the average. However, in many cases all four parathyroids were almost uniform in size. There appeared to be some correlation between the size of the animal and the total parathyroid mass, so that larger bodies might be expected in large individuals.

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[2] I wish to gratefully acknowledge the generosity of Prof. H. D. Reed, who furnished the bullfrogs necessary for an experimental study in connection with the problem of the amphibian parathyroids.

Each parathyroid body is separately inclosed in a rather thick capsule of connective tissue, which is unbroken except where blood vessels pass through it to its inner side. When it is stained with Weigert's resorcin fuchsin, it appears as a continuous deeply stained layer about the parenchyma. With picrofuchsin and other differential stains, the capsule is found to contain numerous fine collagenous-fiber bundles crossing at various angles so that a close mat of white fibers is formed. Between the collagenous fibers are very fine elastic-tissue fibers forming a fine network much less massive than the white tissue. Interspersed between the fibers of the capsule are many connective-tissue cells which are often much elongated and flattened. These cells frequently appear quite thin and spindle-shaped in sections, and are often seen in clumps near the blood vessels. Not infrequently, mast cells and eosinophiles may be found in the capsule. The connective-tissue covering is compact and quite resistant, and may be as much as 6 to 9 μ thick in some instances. Sometimes greater or lesser areas of the capsule are covered by a thick layer of adjoining connective tissue which has been deflected from its course by the presence of the parathyroid body. Such a secondary capsule may be seen in figure 1, which shows a thick layer of connective tissue covering a part of the true capsule and connecting with the wall of the external jugular vein above. This secondary capsular coat does not cover the entire organ, with the result that the capsule may appear much thicker on one side than on the other in a section which has been taken in a plane such that it passes through the capsule proper on one side and through the capsule plus the secondary capsule on the other. Figure 2 is a photograph of such a section showing a thick region in the capsule, due to the presence of a secondary layer of connective tissue. The secondary capsule is frequently much thicker than the capsule proper and may contain blood vessels. Just beyond this outer capsule is a loose reticulum of connective-tissue fibers and fat-cells, the fat being more abundant during the fall and winter seasons. The capsule is similar in structure, but much thinner in R. palustris and R. pipiens than is that of R. catesbeiana.

The parenchyma

The parenchyma of the parathyroid consists of epithelioid cells which make up the greater part of that body. The central region of the organ is composed of almost pure epithelioid cells closely compacted into a more or less spherical mass. When a cross-section is examined, the cells appear to be arranged in cords which have been wound upon one another in spirals and coils and closely crowded together. Figure 3 shows this characteristic feature of the frog's parathyroid. The appearance of the cell cords and the behavior of the cells during growth and regeneration suggest that the cords are the result of the way in which the cells were oriented during the process of growth, and that they represent growth whorls which have been influenced by crowding. The effect produced by the twisting of these growth currents is probably the most striking peculiarity in a section from the anuran parathyroid. When a cord lies in the plane of the section, the cells appear to be long and spindle-shaped and the nuclei are seen to be elongated. If the cord is perpendicular to the section, the nuclei will appear to be round; but since the cords are cut at different angles, the cells and nuclei present all gradations in shape, ranging from the spherical to the elongate type. Such a picture would be possible even though only the elongate type of cells were present; but if the cells are macerated and teased apart, they are actually found to be quite varied in shape. They were found to range in shape from rather smooth elongate cells, through rough polygons, to those having very pronounced arms or processes. Satisfactory preparations for this study were obtained by macerating fresh tissue for a few hours in 0.5 per cent osmic acid and then washing the tissue and teasing it in isotonic sodium-chloride solution. The cells were then studied in salt solution or mounted in glycerin.

The nuclear material is very conspicuous in stained preparations of the frog's parathyroid. The nuclei are large in comparison with the size of the cells and appear therefore very numerous and close together in sectioned preparations of tissue taken during most months of the year. This is well illustrated in figure 3. The exceptions to this general picture will be discussed later. The shape of the nucleus is determined somewhat by the shape of the cell, with the result that some of them are more or less spherical, while others are very much drawn out. The size of the nucleus in relation to the cell as a whole is surprisingly large. A spherical nucleus may have a diameter of 10 to 12 μ, while the diameter of the entire cell may range from 15 to 18 μ. An elongate nucleus may attain a size of 8 to 10 μ in diameter and 17 to 20 μ in length; while the entire cell may not measure more than 9 to 10 μ in diameter, but is sometimes twice the length of the nucleus or more. There are, however, many smaller cells which likewise have proportionately smaller nuclei. Each nucleus is surrounded by a membrane which appears quite distinct in fixed preparations that have been suitably stained. The presence of the membrane is also indicated when the macerated cells are examined, and again when the parenchyma is undergoing a general breakdown which constitutes a phase of the cyclic change to be described later. The nucleolus is quite large and distinct, as seen in fixed preparations. Sometimes two or more nucleoli are present in a single nucleus. The chromatin nucleoli are stained deeply with nuclear stains and may be identified by the use of the common dyes. They are best demonstrated by the use of such chromatin stains as Heidenhain's iron hematoxylin or methylene blue. The nuclear membrane of the resting nucleus is filled with a substance frequently referred to as 'nuclear sap,' which appears quite clear and transparent after the process of differentiation. Penetrating this karyochylema there is a fine network whose threads appear to extend to the nuclear membrane and to connect with the nucleolus. This nuclear network is seen in sectioned preparations after fixation in Zenker's fluid, Helly's fluid, Bouin's fluid, Heidenhain's mixture, or certain other fluids and then staining with eosin-methylene blue, iron hematoxylin, Mayer's hematoxylin, or brazilin. The chromatin is distributed unevenly along the threads of the net in the form of granules and small clumps.

A careful examination of sectioned preparations revealed a difference in the staining reaction of the resting nuclei, some staining somewhat more deeply than others. The darker nuclei have an abundance of chromatin material and a close-meshed network, while the karyochylema holds the stain more successfully during differentiation and remains opaque. In fact, these nuclei are able to take the stain more rapidly, since they stain more deeply with progressive methods. The light and more transparent nuclei, on the other hand, have a clear transparent karyechylema, a more open nuclear net, and the chromatin appears to be somewhat reduced in amount. This difference between the two types of nuclei does not appear to be confined to any special method of fixation and staining. Neither is either of the nuclear types confined to any given area of the organ, but the one may be found in close proximity to the other. There are all gradations between these two extreme types. For this reason and also because of the observed behavior of the nucleus in cyclic changes, which are to be described later, it is considered probable that these differences may represent stages in the life-cycle of the cell. Romeis ('26) describes, in R. temporaria, a nuclear type which stains brownish black with 'Eisenbrasilin' after Zenker's fixation. The writer made similar preparations from the parathyroids of R. catesbeiana, but failed to obtain the nuclear type described by Romeis, where the cells were in a compact and healthy condition. Pictures resembling those described by Romeis were obtained in parathyroids where cytolysis was taking place. The dark-brown nuclei in this preparation were those that were in an early stage of chromatolysis (figs. 7 and 8). Dark nuclei are shown within a mass of colloid and others in regions where the process of vacuolization was in the early stages. The parenchyma cells in R. catesbeiana undergo a seasonal cytolysis which involves the nucleus as well as the cytoplasm, and which will be described later together with the method of cell division found in the parathyroids. It should be pointed out, in this connection, that Romeis did not always find the dark type of nucleus present in his preparations.

The parenchyma cell is poor in cytoplasm. Near the center of the parathyroid the nuclei lie very near one another, being separated by a very thin covering of cytoplasm. Most of the cytoplasm of these cells is drawn out into a long projection at either end of the nucleus and presents an outline which somewhat resembles that of smooth-muscle cells, when cut in a suitable plane. Near the outer border of the gland and also in close proximity to the blood vessels the cytoplasm is noticeably more abundant. Figure 5 shows the central area of a parathyroid in the upper part of the photograph and the border zone of that body below. The cells in the border zone are large and are comparatively rich in cytoplasm. Sections which have been stained in hematoxylin and eosin after acid fixation show fine protoplasmic strands penetrating the cytoplasm. These stain heavily with eosin and are beaded with faintly staining protoplasmic granules. This same protoplasmic structure is brought out by the use of eosin-methylene blue, which stains the granules a light purple. These structures are probably artifacts due to the way in which the proteins of the cytoplasm are precipitated during the process of fixation. The cytoplasm is rich in chondriosomes of a lipoid nature.

Fat-droplets are of frequent occurrence in the parenchymal cells of the parathyroids of many animals. Numerous attempts were made with frozen sections of fresh tissue to demonstrate the presence of fat with sudan III and Herxheimer's scarlet red, but the results were negative in the case of R. catesbeiana. Various osmic-acid stains, such as Flemming's fluid, Benda's fluid, Bensley's fluid, and Altmann's fixation, likewise gave negative results with this species. It is possible that fat-droplets may be found at some stage in the cycle of the parenchymal cells other than those stages examined by the writer. However, it is certain that fat-droplets are not commonly present in this species. Romeis found that fat-droplets which blackened with osmic acid are formed in the parenchymal cells of R. temporaria. The writer found them present in the parenchyma of R. pipiens. Erdheim ('03) made a careful study of this type of cytoplasmic constituent in a number of mammals and found that the occurrence of fat-droplets seemed to vary with the age of the animal in some cases; but he was unable to demonstrate their presence in all species.

An attempt was made to find out whether or not the cells of the parenchyma contained mucus. It was thought that, considering their origin from the epithelial lining of the pharynx, some of the cells might be expected to retain their mucus-secreting potentialities. A study of the preparations which employed the use of the common fixers and stains gave no discernible evidence of such a product. Material fixed in 95 per cent alcohol and stained with mucicarmine gave negative results. All preparations were free from colloidal vesicles. Colloid formation in R. catesbeiana will be discussed along with a description of the changes which occur in the parenchyma during the seasonal cycle.

Blood vessels

The parathyroid in R. catesbeiana is supplied with blood by way of a branch from the external carotid artery, and the efferent vessels empty into the external jugular vein. The afferent vessel usually penetrates the capsule in close proximity to the exit of the veins. In some instances the parenchymal cords are developed in such a way that a depression resembling a hilum is formed where the blood vessels enter and leave the capsule, while in others the contour is quite uniform. After the afferent vessel has entered the capsule, it divides into numerous branches which continue to branch and anastomose and form a closely meshed network of capillaries which surrounds the parenchymal mass and lies just beneath the connective-tissue capsule. The meshwork of the capillaries is frequently so close that only a slight layer of connective-tissue fibers separates the individual capillaries in places, while in other regions small clumps of connective-tissue cells are interposed. The capsule presses the blood vessels very closely upon the mass of parenchymal cells. The capillaries converge and unite to form the veins which carry the blood away from the organ. The vessels of the net differ in size, from very fine capillaries, which are so small that the blood cells must pass in single file, to vessels several times the diameter of a blood cell. The capillaries sometimes anastomose to form rather extensive blood sinuses. The branching network of capillaries and sinuses greatly increases the surface area of the walls forming the blood channels, and likewise increases the surface resistance to the blood flow. This would tend to slow the blood flow through the parathyroid and to increase the time during which the blood would remain in close proximity to the tissue. The vessels are endothelial-lined tubes which are surrounded by a very thin layer of collagenous fibers. The nuclei of the endothelium are somewhat large and protrude well into the lumen of the vessel. These endothelial cells with large protruding nuclei are shown in figure 19.

The distribution of the capillaries is quite varied in R. catesbeiana. In a few instances the blood supply of the parathyroid is confined to a network of capillaries just beneath the capsule and outside of the body of the parenchyma. More often, blood vessels pass from the capillary net into the parenchymal mass. Figure 20 is a photograph of a section taken through the center of the parathyroid, and shows the presence of numerous large and small blood channels. A study of serial sections shows that in some cases one or two large capillaries penetrate the parenchyma and pass through the center of that mass, bringing the blood supply in this way in closer relation to the cells which are farther removed from the surface. Cases of this type have been observed in which the penetrating capillaries branch within the body of the parathyroid and then again unite as they leave the center of the parenchymal mass. In some instances as many as ten channels of communication have been found between the central area of the parathyroid body and the capillary net which covered the surface. These vessels were found to branch and follow tortuous routes through the parenchymal mass. There is a tendency for the superficial vessels to be less closely meshed when there are many vessels penetrating the parathyroid tissue, but the surface capillaries are abundant in every instance. In general, the large parathyroids are supplied with a greater number of penetrating vessels, whereas a very small body from the same animal may have only one or two vessels which penetrate the parenchyma, or it may even be entirely dependent upon the superficial capillaries for its blood supply. In thirty individuals of this species, not an individual was found in which all four parathyroids were free from penetrating vessels; in fact, it is the rule for the blood vessels to penetrate the parenchyma in this species. In R. pipiens, a smaller species, no cases were encountered where the blood vessels entered the parenchymal mass, and the same is true for the very limited number of R. palustris examined.[3] The findings for R. catesbeiana, which is the largest species of frog, will not admit of the generalization made by Romeis ('26), that the blood vessels do not penetrate the parenchymal mass in the Anura, although his conclusion is probably correct for most species.

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[3] It is impossible at the present time to draw any conclusions regarding the likelihood of encountering exceptions to this distribution of blood vessels in either R. pipiens or R. palustris, owing to the small number of cases investigated.

It was pointed out by Romeis that it is difficult to account for the secretion of the deeply buried cells reaching the blood stream, since the only blood supply of the organ is the capillary net just beneath the capsule. It is equally difficult, however, to explain how such remote cells could be properly nourished. Apparently, there is a limit beyond which this indirect method of communication with the circulation cannot pass. In R. catesbeiana we have a case where the growth processes exceed that limit, and here the transportation problem is solved just as it is in the development of other large organs. Blood channels are formed, which carry the circulating fluid within reach of the cells, so that the physiological needs of the organ are met and its functioning is assured.

Collagenous fibers from the capsule often form small trabecula-like projections into the parenchyma at the place where a blood vessel enters the body of the organ. Figure 4 shows such a trabecula of connective tissue extending for some distance into the central mass of the parathyroid. The photograph shows the blood vessel to be cut in two places and accompanied by numerous connective-tissue fibers. A study of serial sections in either direction from the plane of this section shows that the intruding mass of connective tissue does not form a septum, but merely represents a rather large trabecula extending from the capsule and surrounding the blood vessel. Frequently, the connective-tissue fibers of the capsule are only slightly deflected by the presence of a blood capillary entering the parenchyma, with the result that only a very thin layer of collagenous fibers forming the outer hull of the capillary wall appears in the body of the organ. In one or two parathyroids wide bands of connective tissue have been found to penetrate deeply into the organ and form a pronounced connective-tissue septum. Such a connective-tissue septum is shown in figure 6. In this instance a study of serial sections shows that the smaller mass of parenchymal cells is directly connected with the main body of the organ in a very limited area. When extensively branching blood vessels are found in the parenchymal mass, they are frequently accompanied by more or less connective tissue of reticular appearance. The amount of connective tissue to be found in the body of a parathyroid from R. catesbeiana may range from none at all where the blood vessels are confined to the surface—only the very fine collagenous fibers covering the capillaries in some instances—to a heavy and distinct connective-tissue septum in extreme cases. Here again, the generalization made by Romeis ('26) regarding the invariable absence of connective tissue within the parenchymal mass of the parathyroid in the Anura will not apply in the case of R. catesbeiana.

Accessory tissue

Accessory parathyroids in the frog have been reported by Maurer ('88), by Ecker-Gaupp ('02), and by Romeis ('26). In thirty individuals of R. catesbeiana examined during this study, only one accessory parathyroid was encountered, and in between 80 per cent and 90 per cent of these cases the absence of accessory tissue was verified by the physiological test (Waggener, '29). In the instance cited above, two of the parathyroids were held in close proximity by connective tissue and the third was some distance removed. It was necessary to section the material in order to be sure that the first two bodies were inclosed in separate capsules and were not lobes of the same organ. Such an accessory body might easily be an expression of the mechanics of growth or the result of regenerative processes in the cyclic change of that organ in this species, rather than representing an epithelioid development from a pouch other than pouches III and IV. This possibility is strongly suggested by the occasional occurrence of a deep bilobed parathyroid, as shown in figure 6, and also by the way in which the parenchyma is regenerated after seasonal cytolysis, as illustrated in figures 12, 13, 14, and 18. An accessory parathyroid, formed as a result of being 'pinched off' from the main mass or by a regenerative center of growth becoming isolated from the others, may be regarded as an accessory parathyroid of secondary origin, in counterdistinction to one which might have originated from a separate gill pouch—the latter an accessory parathyroid of primary origin. It would then follow that the presence of an accessory parathyroid would not prove anything regarding the developmental behavior of the gill pouches. The possibility of pouches other than III and IV developing parathyroid tissue would be a question for the embryologist alone to decide. The fact that physiologists and others report the presence of accessory parathyroids far in excess of the number of cases reported by the students of embryology suggests very strongly that accessory parathyroids may develop secondarily, or that some of the accessory parathyroids which have been reported are not parathyroids at all. In regard to the frog, Gaupp ('02, S. 215) states that occasionally the number of epithelial bodies is greater than two. Maurer reported in a few cases—one found in the frog after metamorphosis—three parathyroids on either one or both sides. Romeis found, upon the examination of about fifty individual frogs of European species, four cases of accessory parathyroids.

Cyclic changes in the parathyroid

Cross-sections of the parathyroids removed from R. catesbeiana during the fall and early winter present a picture of closely packed parenchymal cells, as shown in figure 3. The cells are arranged in cords which are twisted and pressed one upon the other. This cell arrangement, together with the fact that each cell has a large nucleus and a scant amount of cytoplasm, gives a crowded appearance to the parenchymal tissue. Such is the type of cell arrangement which was originally described for the frog's parathyroid and is the condition which Romeis describes as prevailing during the greater part of the year in R. temporaria. He found, however, that in this species the closely compact structure was broken down in the late winter and early spring and was later reestablished by cell division. The cells underwent a change which caused them to appear to be full of vacuoles after they were fixed, sectioned, and stained. As a result of this appearance of the sectioned preparations, he spoke of the change as a vacuolization of the cytoplasm. These cytoplasmic changes continued in R. temporaria until the parenchyma had resolved itself into an open network of protoplasmic threads. In the meantime, the nuclei had also undergone degenerative changes and had become greatly reduced in number. These changes culminated in March, and by the latter part of March nuclear division had begun to greatly increase the number present. The crowded condition was rapidly produced, and persisted until the following year.

The parenchymal tissue of R. catesbeiana was found to undergo a cyclic change, but one which differed in many respects from that described by Romeis for R. temporaria. Tissue removed from R. catesbeiana during the early part of December (fig. 3) showed the typical compact condition of the parenchyma. Parathyroids removed about the middle of February and fixed in Carnoy's 6-3-1-sublimate mixture and stained with hematoxylin and eosin showed areas in which the cytoplasm of the parenchymal cells had begun a process of degeneration. With this treatment, a part of the cytoplasm was dissolved and the cells appeared vacuolized in stained sections. The early stages of this process may be seen in figure 10, which shows an area in which the cytoplasm has begun the process of cytolysis. A study of preparations which were fixed in Zenker's fluid, Carnoy's fluid, or most of the other common fixers, dehydrated in alcohol, and embedded in paraffin suggests that the cytoplasm first undergoes a process of liquefaction and that this liquefied substance is dissolved out during the treatment outlined above, with the result that the cells show clear areas when they are sectioned and stained. This phenomenon may be conveniently referred to as vacuolization, but the use of this term should not be interpreted to mean that the clear spaces appearing in the sections were empty spaces in the cell before fixation. As cytolysis continues, the vacuoles appear larger and more numerous and the process comes to involve more and more of the parenchyma. In sectioned preparations fixed in Zenker's fluid and stained in hematoxylin and eosin, the cytoplasm at this stage appears to be reduced to a few processes and protoplasmic threads which may have faint eosin-stained granules along their length. After the cytolysis has continued a little farther, the picture is that of an open net of very fine protoplasmic strands which stain faintly with acid stains. In the meantime, the number of nuclei has become greatly reduced, and, frequently, in the center of such an area no nuclei are to be encountered. This stage corresponds closely to the culmination of the degenerative changes described by Romeis for R. temporaria. But, in R. catesbeiana, the cytolysis does not stop here. The cytoplasmic strands liquefy and large open spaces appear in the preparation—spaces which were formerly occupied by hundreds of parenchymal cells. Eventually, all of the original cells are included in the liquefaction, save a layer of cells probably not more than one cell thick lining the capsule. The tissue was not blackened when it was fixed in mixtures designed to preserve and stain oil droplets, such as Altmann's fluid, Benda's fluid, Bensley's fluid, or weak osmic acid. This shows that the content of the vacuole is not a fatty acid, and that the process is not one of fatty degeneration. Tissue which was in the process of cytolysis was given a short fixation in Bensley's fluid, rapidly dehydrated in alcohol, cleared in oil, and embedded in paraffin. Sections stained with acid fuchsin and methyl green showed the cells to be granular, the granules being fuchsinophile in character. Similar granules were obtained from the substance which was completely broken down. Tissue which was in an advanced stage of cytolysis was given a short fixation in Helly's fluid, passed rapidly through the alcohols, and embedded in paraffin. The sections which were passed through 0.5 per cent potassium dichromate and stained with acid fuchsin and methyl green showed the decomposed area to be filled with a dense and deeply stained colloid. Figure 12 is a photograph of a section from this preparation.

The nuclei are included in the process of cytolysis. It appears that different processes are involved in the nuclear breakdown. Sometimes changes are observed which suggest that the nucleus is destroyed by a process of fragmentation. The karyochylema diffuses out into the cytoplasm and allows the nuclear membrane to shrink and wrinkle. The chromatin figure characteristic of the resting nucleus is broken down and the chromatin appears to be held back for a time by the membrane. This and possibly the liberation of some nucleic acid within the nucleus are probably responsible for the deep staining met with in such instances. As the process continues, the nuclear material becomes quite irregular in outline and finally breaks into fragments. This process of fragmentation is later followed by a liquefaction of the particles. Romeis found this type of degeneration occurring in R. temporaria. This is not, however, the usual type of nuclear degeneration encountered in R. catesbeiana. The method most often met with is a type of chromatolysis in which the chromatin content of the nucleus becomes greatly reduced and finally disappears by a process of liquefaction. During the early stages, the resting nuclei appear transparent and glassy when fixed in Zenker's fluid or Carnoy's fluid and stained with hematoxylin and eosin. The nuclear membrane remains stretched for a time, while the general shape of the nucleus may become more rounded. In advanced stages of chromatolysis the nuclei are small, as a rule, although the membrane may not show much wrinkling. This suggests that possibly some of the nuclear material has diffused out into the cytoplasm and that the membrane has shrunken rather uniformly. During the last stages in the breakdown, the nucleus becomes quite transparent and can be differentially stained only with difficulty. It appears to have lost the ability to take and retain the basic stain. The presence of small clumps of chromatin can be demonstrated in rather late stages of a degenerating nucleus by means of the iron-hematoxylin stain. This and also the fact that degenerating nuclei fixed in osmic-dichromate fluid and stained in acid fuchsin show the nuclear material to be rather strongly fuchsinophile suggest that, along with the liquefaction of the nucleus, the basichromatin may possibly be converted into oxychromatin. In advanced stages of the degeneration the nucleus may hold the cytoplasmic stains. In the last stages of nuclear disintegration, after Zenker's fixation, the nuclear membrane may remain filled with an opalescent substance which is quite indifferent to certain of the common nuclear stains. The membrane is finally dissolved and the identity of the nucleus is lost. When the cells are fixed in Zenker's fluid, the nuclei often shrink, so that many bizarre forms are encountered in sectioned preparations. With the collapse of the nuclear membrane and the retention of the nucleolus and more or less chromatin, the nuclei may often stain quite darkly. Zenker preparations frequently show some dark-brown nuclei when stained with brazilin. These densely staining nuclei are apparently in the process of disintegration. Degenerating parathyroids fixed in Zenker's fluid show a large number of such pycnotic nuclei, which are more abundant in the outer border of the parenchyma and are less often encountered near the center. This distribution applies particularly for the early stages in the autolysis of the organ. Some of these pycnotic figures are probably the result of the treatment to which the tissue has been submitted, since they are rare in other preparations which are equally degenerate but differently treated. However, both deeply staining and transparent nuclei may occur side by side at the very border of a liquefying area. Both types of degenerating nuclei may be seen in figures 7, 8, and 9. The nucleolus remains intact, even when chromatolysis is in its last stages. This is not apparent in all preparations, but in most instances the nucleolus is observed to remain within the nuclear membrane and to stain deeply even when it is difficult to otherwise distinguish the nucleus. In certain preparations the nucleolus appears to be able to retain its identity even after the cell has disintegrated. The compact and crowded appearance of the cells of the parenchyma is lost soon after the cytolysis has begun. While the direction of the cell whorls can be determined for some time, they are by no means so pronounced as they were before the onset of degeneration, and they are eventually obliterated as liquefaction proceeds.

The connective-tissue capsule remains intact and retains the colloidal mass which is formed by the liquefaction of the parenchyma. Within the capsule and lining it, is a layer of cells which does not liquefy with the remainder of the parenchyma. The capillaries beneath the connective-tissue capsule do not appear to be fully distended at the time when the cytolysis of the parenchyma is nearing completion. Sectioned preparations of the parathyroid at this stage showed that the capsule had not collapsed. The distended capsule is shown in figures 12 and 13. The parathyroid body appears to be quite normal at the time of removal during this season of the year. It retains its shape and is distended in most if not all instances. Successful fixation and embedding, so that the colloid is retained and the capsule held distended, are not always attained. In most instances the mass shrinks during the process, and the capsule appears somewhat wrinkled and collapsed when it is sectioned. The normal appearance of the parathyroid at the time of removal, together with a few cases of successful preservation, indicates rather clearly that the capsule retains the liquefied mass and does not collapse during the time when the parenchyma is being dissolved.

The nature of the colloid which is formed as the end product of liquefaction is probably quite different from the substance formed during the early stages of vacuolization. That the content of the vacuoles is not a fat is indicated by the negative osmic-acid treatment mentioned above. The fact that it is lost by fixation in Zenker's fluid and other acid fixers shows that the content of these spaces is not a typical colloid. In describing the vacuolization which occurs in R. temporaria, Romeis ('26) has said that the content can be demonstrated by no known method. The fact that the content is not preserved by ordinary fixation indicates a substance which contains little or no protein. Tissues from the bullfrog, removed on February 28th and fixed in Zenker's fluid for three hours, passed rapidly through the alcohols, and embedded in paraffin, show the presence of a colloid in the liquefied areas, which stains faintly with hematoxylin and eosin. Brazilin gave a deeper stain with this preparation, while Mallory's connective-tissue stain colored the colloid deeply. Figures 7 and 8 are photographs of a section from this preparation, which was stained with brazilin, and show areas of colloid within the body of the parathyroid and near the blood vessel. Figure 9 is a high-power view of a section from the same parathyroid stained with Mallory's connective-tissue stain. A stiff colloid is preserved in the area where liquefaction is complete, but the vacuoles in the adjoining cells are empty. The colloidal mass has been observed to border the vacuoles and to extend out along the region of the protoplasmic fibers of the cytoplasm. Colloid processes and branches have also been observed to project from the central mass and to extend out between cell vacuoles and along the cell borders. This suggests that it is the final cytolysis of the protoplasmic strands and the cell walls which supplies the protein element of the colloid in such preparations. The nuclei might conceivably contribute materials to the liquefied mass, which would be precipitated and preserved; but, if so, it must be a minor part. Many nuclei may disintegrate in the periphery of the parenchyma, so that the nuclei are very sparse, and yet the cytoplasm will present very marked vacuolization without any trace of colloid formation. Wherever the colloid was encountered, complete liquefaction of the cells was in progress or had at least involved the cytoplasmic fibers and the cell borders.

The best preservation of colloid (fig. 12) was obtained by fixing the tissue for two and one-half hours in Helly's fluid, passing it into Müller's fluid for an equal time, washing in water, dehydrating in alcohol, clearing in cedar-wood oil, and embedding in paraffin. The sections were given a short chromation in 0.5 per cent potassium dichromate and stained with acid fuchsin and methyl green. In this preparation a very dense, homogeneous, deeply staining colloid was demonstrated. The parathyroid from which the section shown in figure 12 was taken had undergone almost complete cytolysis. Only a very small fragment of the old cells yet remained. Sections from this same preparation were stained with iron hematoxylin and orange G, and the colloid also took this stain. Sections stained with eosin and methylene blue, without special chromation, gave only the faintest evidence of the presence of a colloid, which appeared as a glassy transparent mass. Tissue fixed in Benda's fluid and given a short chromation before staining with acid fuchsin and methyl green showed the end product of cytolysis to be preserved in the form of a flocculent precipitate of fine and coarse granules which were strongly fuchsinophile. The same granular effect was also obtained by staining this preparation with iron hematoxylin and eosin, the granules being a faint pink in color. The remnants of this flocculent precipitate may be seen within the old capsule, as shown in figure 13. This organ was in approximately the same stage of cytolysis as was the parathyroid shown in figure 12. Only a very small fragment of the old parenchyma remained undissolved, and the process of regeneration was well under way; both of which conditions were equally true for the case illustrated in figure 12. The stage in the cycle may, therefore, be disregarded in the comparison of these two parathyroids, since it is the same in both instances. With the stain eliminated by trial, this leaves only the process of fixation to account for the difference in the two colloids. Fixation in Benda's fluid and chromatin is a method designed to preserve the chondriosomes, particularly those of a lipoid consistency. After this fixation, the degenerating cells are granular and the colloid is likewise granular. This would suggest that the colloid was not originally homogeneous in its consistency, and that the first steps in the liquefaction of the cells of the parenchyma were probably concerned with the liberation of certain lipoid-like substances. These substances, by their very nature, might be expected to be lost with such fixers as Zenker's fluid, Carnoy's fluid, etc., and, more especially, if fixed for a long period.

It is rather to be expected that the end point of cytolysis would be the formation of a colloid. It should be pointed out, in this connection, that the colloidal nature of a substance does not necessarily identify it with any other thing in the colloidal state. It might be that the only constituent which two colloids possess in common is protein. This is far from saying anything very definite, because there is a large variety of proteins. Colloidal vesicles have been reported in the parathyroids of certain animals, including man, and various attempts have been made to associate this colloid formation with the hormone-producing activity of the gland. Since the thyroid is very rich in colloid and for other reasons, it is held by some that the colloid may be the carrier of the thyroid hormone. Whether or not this interpretation is correct lies outside the bounds of this discussion. But such considerations have led many investigators to turn to the parathyroid for a similar colloidal carrier of the parathyroid hormone. The discouraging feature of such research was the fact that colloid was not to be found in the parathyroids of certain animals. Whether or not this seasonal colloid formation in the frog has anything to offer in helping to understand the secretory nature of the tissue, awaits further investigation. It is apparent, however, that the colloid formed by the breakdown of the parathyroid is different from that encountered in the thyroid; in fact, it differs from most colloids. This is evidenced by the difficulty with which it is retained in fixed preparations. It appears that acid fixers and slow processes tend to dissolve out a large portion of the substance, so that only shreds and fragments of precipitated protein remain. Hence, large open spaces may be encountered where complete cytolysis has taken place. No such difficulties are encountered in the preservation of the colloid of the thyroid, for example. This behavior of the colloidal mass of the parathyroid might be interpreted as indicating a protein-poor substance, which is in keeping with the already-observed fact that parathyroid cells of the frog have a very small ratio of cytoplasm. Again, this scant protoplasm is very rich in chondriosomes, as pointed out by Romeis, who comments on their surprisingly large number. These observations, together with the behavior of the colloid, seem to support the conclusion that the end product of cytolysis is poor in proteins and rich in lipoids or substances which behave like lipoids.

The process of regeneration in the parathyroid of R. catesbeiana was observed to begin some time before all of the old parenchyma had completely liquefied, as illustrated in figure 13. The parenchymal tissue is replaced by rapid cell divisions which occur in certain localized areas or growth centers. These growth areas have their origin along the inner border of the capsule and develop outside of, and in connection with, the thin layer of parenchyma cells which lines the capsule. As the new tissue grows, it pushes this border membrane ahead of it in such a way that a thin cellular membrane incloses the growing cords of parenchyma cells and separates them from the colloidal content within the capsule. The point where such growth areas originate is usually in close proximity to a blood vessel. Just what initiates this active growth is not known. What determines the exact location of the growth centers is not clear, although it is possible that a convenient exchange of nourishment and waste disposal are important factors. Both the number and position of these growth areas probably depend upon some local or intrinsic condition. The least possible number of such areas would be one, and no such instance was encountered. In many cases ten to twenty growth centers were found. The examination of one regenerating parathyroid showed many more—indicating that regeneration might set in at almost any point along the extent of the surviving membrane. Figure 13 shows a large mass of regenerating tissue which extends well into the lumen of the old capsule and which has developed about a number of branching blood vessels. This large parenchymal mass has eight points of contact with the old capsule, and each isthmus of tissue carries one or more blood vessels which branch in the center of the main mass. Incidentally, this conveys some idea of the large number of blood vessels which may be found within the body of the parathyroid. The surviving membrane which lines the old capsule is continuous along each isthmus and completely surrounds the regenerating mass. This membrane consists of deep columnar cells about the isthmus. Figure 17 shows a section of the membrane near the isthmus. In either direction from the isthmus, these cells gradually merge into the cuboidal type and finally into a very thin pavement epithelium which forms the limiting membrane about the greater part of the regenerating mass. Figure 15 shows a section through the membrane surrounding the regenerating body. The photograph is confined to an area not far from the isthmus, and shows the cuboidal type of cells. Figure 16 shows the same type of membrane cells lining the old capsule at a point a short distance from the isthmus. The cytoplasm of the columnar and cuboidal cells stains deeply with eosin, and appears granular. In places along the membrane, a striated border is apparent. The colloid which filled the capsule is slowly reabsorbed as regeneration continues. Phagocytic cells and lymphocytes are encountered in the blood channels. Mast cells and eosinophiles have been observed in the connective tissue. Figure 12 shows one large cell cord protruding into the lumen of the capsule and four other cell cords developing between the surrounding membrane and the old connective-tissue capsule. Figure 18 shows, in addition to the one large regenerating body, three or four small cords of regenerating tissue developing from growth centers of a later origin. Figures 13 and 14 show, in addition to the one large regenerating body, a cord of cells which has extended into the connective tissue of the capsule, instead of protruding into the lumen. A study of serial sections reveals two other cords of a similar type located in contact with this one, but at a different level from the plane of the section shown in the photograph. The connective-tissue capsule may be seen forming about this cell clump, and it is possible that, as growth continues and the entire mass becomes one compact body, such groups of tissue may grow together in such a way that deep connective-tissue septa will remain after full regeneration. An accessory parathyroid of a secondary origin might conceivably arise as a result of one such growth center becoming completely encapsulated by connective-tissue cells. A capsule of this type would prevent the solidification of the entire mass of parenchymal cords into one body.

Cell division by mitosis is very much in evidence during the period of rapid growth. Mitotic figures are encountered both in the membrane cells and within the cell mass. From two or three to ten or more mitotic figures were found in each section of a regenerating mass, which was about 250 to 300 μ in diameter. This would seem to indicate that mitotic cell division is sufficient to account for the rapid increase of parenchymal cells. Mitotic figures are more abundant in close proximity to blood vessels. The active division which occurs in the growth centers causes some of the cells to be crowded into less favorable positions. Growth and cell division are retarded in these less favorably environed cells. Mitosis is seldom encountered when the parathyroid is in the compact stage. In R. temporaria, Romeis describes the center of growth and nuclear division as being located in the center of the open reticulum. The continued cell division crowds the cells toward the capsule. Further cell division results in deflecting the cells in such a way that the cord-like appearance is the result, and the crowded, compact condition of the parathyroid is finally reestablished. In trying to explain how the cells of the parenchyma, which were buried fifty or more cells from the capillary network, were able to function as secreting bodies, Romeis conceived of the central location of the growth center as of primary importance. Pointing out that in the peripheral portion the cell divisions are less numerous and the cytoplasm richly provided with inclusions is better developed, he suggests that it is in the outer zone that the secretory activity is most pronounced. The immediate proximity of the blood vessels thus facilitates the removal of the elaborated secretion which would be liberated in part at least through the degeneration of cells, which is a marked feature of the peripheral zone. Replacement of such cells would be accomplished through the cell cords, which, pressing toward the surface, would always provide new cells for the peripheral zone. In such a manner Romeis conceives that the cellular material of the anuran parathyroid is subject to a continuous slow transportation toward the surface.

The above interpretation is very interesting, in so far as it is an attempt to account for the way in which the hormone reaches the blood stream under the anatomical relations described for R. temporaria or other species which possess parathyroids of the same general structure. In R. catesbeiana, however, the relationship is a different one. In the first place, the blood vessels are not confined to the surface of the parenchymal mass. Again, the growth areas are not centrally located with regard to the inclosing capsule, but are variously distributed about its border, as was pointed out above. Both the points of origin for the growing cords and the areas in which mitotic figures are most abundant indicate that the growth centers are near the blood vessels and that the cells are not crowded from the growth areas toward the blood vessels, but away from them. The fact that in this species, in which the parathyroid develops to a large size, the blood vessels penetrate the parenchyma suggests that when the accessibility of the blood supply really becomes a problem, it is met in the customary way—blood channels are formed within the organ. Moreover, it seems entirely possible that the products of secretion might reach the blood stream as easily as could the by-products of growth. What would appear to be of prime importance for a healthy tissue is an adequate exchange of foodstuffs and waste materials. A shortage of the former or an excess accumulation of the latter could not be tolerated to an extreme degree. The relationship between the growth centers and the blood vessels in R. catesbeiana might be interpreted as emphasizing this need. Such an interpretation would also be in accord with what is commonly known to occur in connection with the growth of epithelial cells, namely, that the growing and reproducing cells rest upon a substratum of vascular connective tissue. In this event, it seems likely that the explanation of the relationship between the parenchyma, connective tissue and the blood-vascular system of the parathyroid lies in the problem of growth and the mechanics of development.

This unusual cycle of changes, from the compact structure of the parenchyma through the various stages of cytolysis to the fully regenerated organ, transpires in a comparatively brief period of time. Four parathyroids removed from an individual of the species R. catesbeiana on November 21st showed that the complete set was in the compact stage. Tissue was removed from several individuals of the species R. pipiens in December, which presented varied pictures of vacuolization. In the instance of a few bodies removed at this time, the number of nuclei had become greatly reduced. The parathyroids were removed from an animal (R. catesbeiana) on February 7th, and all four parathyroids were found to be in the compact stage. One of the bodies showed that vacuolization of the cytoplasm had taken place in only a very few cells, while a second showed much more vacuolization. A section from this latter body is shown in figure 10. The other two bodies showed no indications of cytolysis. The parathyroids removed from an animal on February 14th showed pronounced vacuolization in two of the bodies, one of which had a large area that had almost entirely broken down (fig. 11). A third body showed vacuoles appearing in a few of the cells, while the fourth had not begun the process of cytolysis. The parathyroids removed from a second animal at this time indicated that they were undergoing the same process. One of them showed only slight vacuolization in a few cells, while the other had a large area of degeneration similar to that shown in figure 11. The parathyroids removed from an animal on February 27th showed a small degenerative area in one of them and a slight vacuolization in another, while the other two were apparently quite normal. The parathyroids removed from an animal on the following day showed marked degeneration. Two of the parathyroids contained only strands and small clumps of cells, while a third body showed large degenerative areas. The fourth body was just beginning to show vacuolization in small areas. Tissue removed from other animals during the early part of March was found to be in almost the same stages of cytolysis. Tissue removed on April 6th showed one parathyroid which had partially regenerated (fig. 13). This particular organ has been previously described. A second body had almost completely regenerated, while the third was about two-thirds liquefied and the fourth was beginning to liquefy. Tissue removed from an animal on April 30th showed more complete regeneration. In two of the parathyroids cytolysis was completed, in the third body only a slight fragment of old cells remained, while in the fourth there was a slight area that had not undergone cytolysis. The regenerated mass filled about three-fourths of the old capsule in each of two of the parathyroids, while in the other two the new tissue was equal to about one-half and one-fourth of the original mass. Figures 12 and 18 show sections taken from two different parathyroids which had been removed on April 30th. Tissue removed on June 18th showed complete regeneration of all four parathyroids.

From the observations just reviewed, it is evident that there may be some individual variation in the progress of degeneration and regeneration. This is illustrated by the tissues removed from different animals, for example, on or about the last of February and the first of March. The tissue removed from an animal on February 27th showed less cytolysis than had occurred in two other cases by the middle of February. In no instance was the cytolysis found to occur simultaneously in all four parathyroids from the same animal. Frequently, one or two of the parathyroids would be almost completely broken down before the last one showed any very definite indications of cytolysis. In all observed cases, moreover, the process of regeneration was well under way before the cytolysis of the old tissue was completed.

The amount of healthy parenchymal tissue present at any one time during the process of change probably did not drop below one-half, and certainly did not drop below one-fourth, of the total amount originally present. Taking into account the fact that the parathyroids are probably vital organs in all animals which possess them, it would seem plausible to expect that the timing of events in such a way that a rather large portion of the parenchymal tissue is present at all times may have a physiological significance and that it is not merely an incidental or chance result of a series of events. Parathyroidectomy of these frogs showed that they were dependent upon the parathyroids during the period of cytological change (Waggener). This cytological cycle in the parathyroid appears to occur in all individuals of this species during the late winter and early spring months. It should, however, be mentioned in this connection that these animals had been transported out of their normal habitat.

The reason for this cyclic change is not known. One obvious result of this behavior of the parathyroid is that a full quota of young parenchymal cells is provided each spring. The season of the year during which this change takes place is approximately that during which certain other seasonal changes transpire. Regenerative changes, somewhat similar to that just described for the parathyroid, have been reported by other workers in the instance of certain other organs of a glandular nature in the Anura. The parathyroid is undergoing its cytological changes at a time when the gonads are active. Whether or not there is a cause-and-effect relationship between the behavior of the parathyroid in the frog and the preparation for the rutting season, is not known. Furthermore, this pronounced change in the cycle of parathyroid tissue occurs at a time when the animal is becoming active and the general metabolism is being hastened. Neither is it known whether there is any causal relation between these two cyclic events. It will require investigation of an experimental nature and an extensive histological survey before much light is shed on the possible relationships mentioned above.

The duration of the winter sleep might be expected to have some effect on the parathyroid tissue. The slowing of metabolic activity might conceivably result in stagnating the deeply buried epithelioid cells of the parenchyma. What seems more likely is that the cells have become exhausted during the period of hibernation and that they are ready to be largely replaced when the spring awakening occurs. As has been previously pointed out, the parathyroid gland is doubtless functioning during the winter season, inasmuch as parathyroidectomy is attended by the death of the frog during this period. It may easily be possible that no one of these seasonal changes influences the other, but that they are each different expressions of some common cause. The organism as a whole is, however, influenced by the changes which take place in its environment, and the cyclic changes which occur in the frog's parathyroid might conceivably be an expression of this animal's response. In such an event, the explanation of this unusual cellular phenomenon will involve both the external conditions of the environment and the intrinsic nature of the organism.

CONCLUSIONS AND SUMMARY

1. The parathyroids in Rana catesbeiana are commonly four separate and discrete little bodies. They are each separately inclosed in a rather thick and compact connective-tissue capsule, which consists of collagenous and elastic fibers and connective-tissue cells.

2. The parenchyma of the parathyroid is composed of epithelioid cells and comprises the bulk of the organ. The cells are arranged in cords which are twisted and closely crowded, giving the organ a compact appearance in cross-section. The cell itself is characterized by a very large nucleus and a scant amount of cytoplasm.

3. The negative results obtained from histochemical tests carried out on the parathyroids of R. catesbeiana support the view that the fat-droplets, colloid, and mucus, when they occur in parathyroid tissue, have no connection with the hormone-producing activity of the organ.

4. The blood-vascular system consists of a fine network of capillaries, which surrounds the parenchyma and lies just beneath the capsule. Branching capillaries commonly penetrate the parenchyma in R. catesbeiana, and they are frequently accompanied by more or less connective tissue.

5. The relationship which exists between parenchyma, connective tissue and the blood-vascular system in the parathyroids of this species does not support certain generalizations made by Romeis ('26) concerning the parathyroids in the Anura. The points of difference are presented in this paper.

6. The parenchyma undergoes a very curious cycle of changes in this species. The cytoplasm of the cell is decomposed by a process of cytolysis which first causes the cell to appear to be vacuolized after acid fixation. The nuclei are broken down by chromatolysis and pycnosis, and the entire parenchyma, except for a layer of cells lining the capsule, is finally liquefied. During a stage of this cytolysis, the products of the liquefaction may be preserved and demonstrated to be in the form of a colloid.

7. The colloid which is formed by the degenerating parenchyma is of a unique character, and differs in its behavior from the colloid which is found in the thyroid vesicle.

8. The disintegrated tissue is replaced by young cells which are developed in rather definite growth centers. These growth processes begin some time before cytolysis is completed, and continue until the entire organ is restored. The centers of growth have their origin along the inner border of the capsule. The growing tissue develops behind the layer of cells which lined the old capsule and which failed to break down during cytolysis. Mitotic figures are very common during the period of rapid growth and regeneration.

9. This cycle of changes was observed to begin about the first part of February, and regeneration appeared to be completed, in this species, by the last part of May or the first of June.

10. The cytolytic and regenerative processes of the parathyroid transpire in such a way that the amount of apparently 'normal' tissue present at any one time is a rather large portion of the total mass of the parenchyma commonly present.

11. The parathyroid performs an important physiological function in R. catesbeiana during this period of profound cytological change. This suggests that the correlation between cytolysis and regeneration has a physiological significance, and is not merely a coincidence.

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[4] For references not listed in this bibliography, the reader is referred to the author's paper entered below.

PLATES

PLATE 1

EXPLANATION OF FIGURES

1 Photograph of a section of the parathyroid from R. catesbeiana, which had been fixed in Zenker's fluid and stained with brazilin. This section shows a secondary capsular layer of connective tissue which extends from the wall of the external jugular vein to the parathyroid body and covers a part of the capsule proper of that organ. × 304.

2 Longitudinal section through the parathyroid of R. catesbeiana. The tissue was fixed in Heidenhain's mixture and stained with Mallory's connective-tissue stain. The section passes through the secondary capsule, which appears as a thickened area on the capsule proper. × 76.

3 Section from a parathyroid (R. catesbeiana) fixed in Zenker's fluid during December and stained with Mayer's haemalum and picrofuchsin. The cells are closely packed and arranged in cords. The large nuclei and the scant amount of cytoplasm give a crowded effect. × 154.

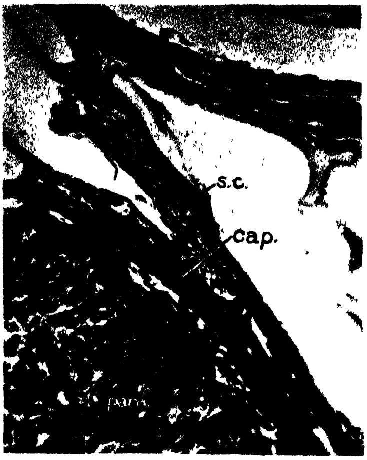
4 Section through the center of a parathyroid which was fixed in Zenker's fluid, February 18th, and stained with Mayer's haemalum and picrofuchsin. The photograph shows a trabecula of connective tissue at a point where blood vessels enter the parenchyma. A vessel may be seen to lie in the plane of the section for some distance. × 304.

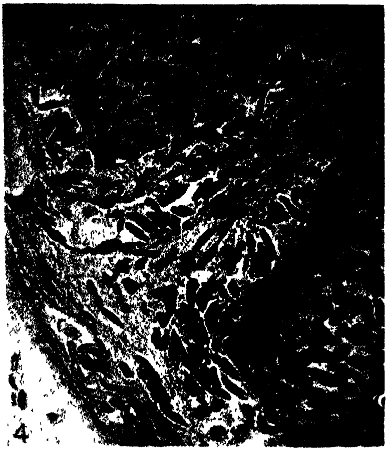
5 Section of a newly regenerated parathyroid of R. catesbeiana. Fixed in formalin, June 18th, and stained with Mayer's haemalum and eosin. The crowded appearance of the central area of the normal parathyroid is shown in the upper part of the figure. Large cells which have a more abundant cytoplasm may be seen near the surface of the parathyroid mass, in the lower part of the picture. The blood vessels which,are shown at the lower margin of the photograph are a part of the capillary net which lies just beneath the capsule. × 304.

6 Section of a parathyroid (R. catesbeiana) fixed in Carnoy's fluid, February 18th, and stained with haemalum and picrofuchsin. This photograph illustrates a connective-tissue septum which separates a small body of the parenchyma from the main mass. × 154.

ABBREVIATIONS

e.j.v., external jugular vein	*para.*, parathyroid
cap., capsule proper	*s.c.*, secondary capsule

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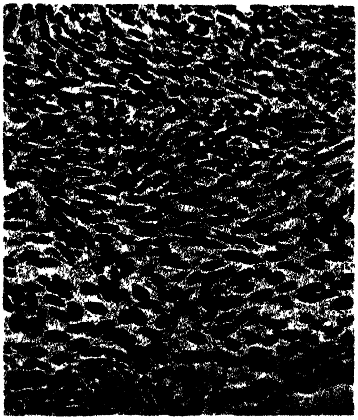

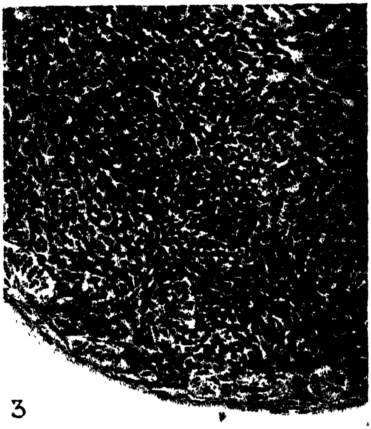
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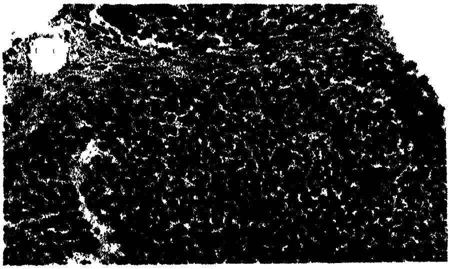


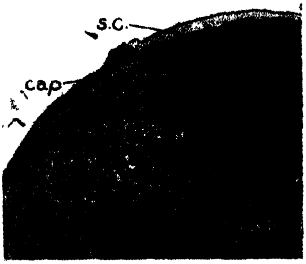


PLATE 2

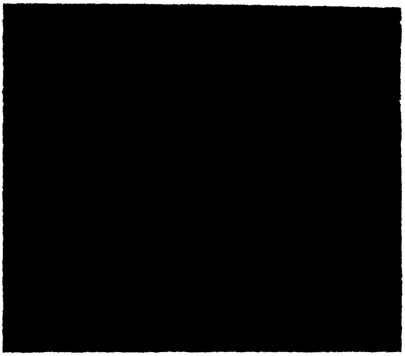
EXPLANATION OF FIGURES

7 and 8 Section of a parathyroid (R. catesbeiana) fixed in Zenker's fluid, February 28th, and stained with brazilin. Cytolysis has proceeded to the extent that rather large areas are completely broken down, and colloid is seen to fill these spaces. Dark-brown nuclei may be seen in the areas of marked disintegration. Both dark and light nuclei are present in this preparation. Figure 7, × 154. Figure 8 (same as fig. 7), × 304.

9 Section of a parathyroid (R. catesbeiana) fixed in Zenker's fluid, February 28th, and stained with Mallory's connective-tissue stain. This photograph illustrates the formation and distribution of the colloid in the process of cytolysis. The colloid is present in areas where the cells have completely broken down and also along the borders of other cells. × 600.

10 Section of a parathyroid (R. catesbeiana) fixed in Heidenhain's sublimate-trichloracetic-acetic acid mixture, February 7th, and stained with Brillantschwarz 3 B and carmine. This photograph illustrates an early stage in the cytolysis of the parenchyma, vacuolization having begun in a limited area. × 304.

11 Section of a parathyroid (R. catesbeiana) fixed in Heidenhain's sublimate-trichloracetic-acetic acid mixture, February 14th, and stained with Brillantschwarz 3 B and carmine. This photograph shows an area which has been completely liquefied, except for a few cells which have not completely disintegrated. × 304.



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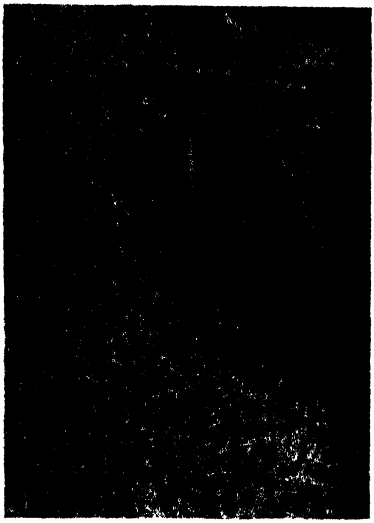


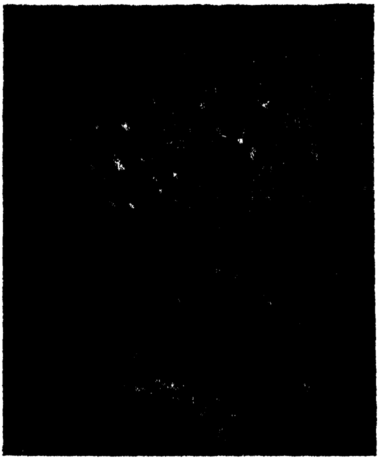


PLATE 3

EXPLANATION OF FIGURES

12 Section of a parathyroid (R. catesbeiana) fixed in Helly's fluid and Müller's fluid, April 30th, and stained with $K_2Cr_2O_7$, acid fuchsin, and methyl green. This photograph shows the old capsule filled with a dense colloid, a regenerating mass protruding well into the old capsule, and other growth centers of later origin. × 68.

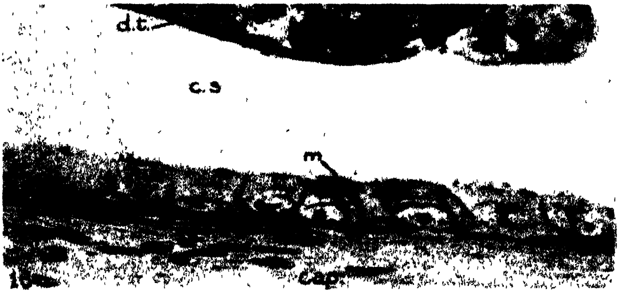
15 Section through the regenerating mass which is shown in figure 13, but at a slightly different level. Fixed and stained as described for figure 13. This photograph shows an area which is located a slight distance from the isthmus, and features the cuboidal cells of the membrane which surrounds the regenerating mass. × 600.

16 Photograph of the same section as figure 15, but showing an area along the old capsule a short distance from the isthmus. This figure features the cuboidal membrane cells lining the inside of the old capsule. A fragment of old tissue which has not yet disintegrated is shown in the colloidal space. × 600.

17 Photograph of the same membrane and section as that shown in figure 15, but showing an area very near the isthmus. This figure features the columnar cells of the membrane which surrounds the regenerating mass. × 680.

ABBREVIATIONS

cap., old capsule
c.s., colloid space
d.t., degenerating tissue
r.m., regenerated mass
g.c., growth center
i., isthmus
m., membrane cells
e.n., endothelium nucleus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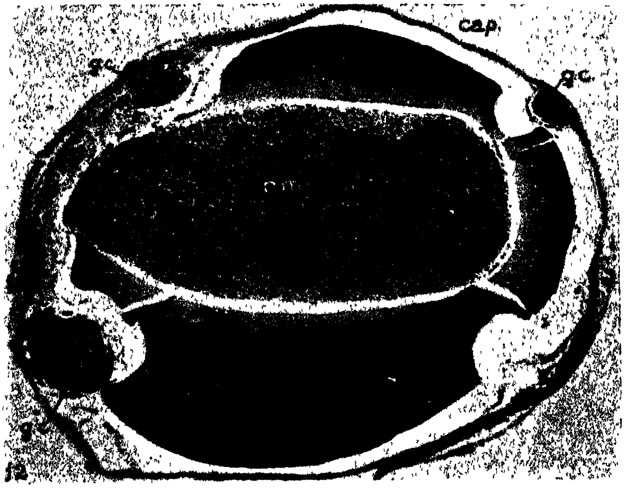
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12

PLATE 4

EXPLANATION OF FIGURES

13 Section through the parathyroid (R. catesbeiana). This organ was fixed in Benda's fluid, April 6th, and stained with iron hematoxylin and eosin. The photograph is of a section taken through the old capsule. It shows a flocculent precipitate within the cavity of the old capsule, a regenerating mass within the cavity of the capsule, an isthmus connecting the regenerated body with the old capsule, and a fragment of degenerating tissue which had not yet liquefied. The entire capsule is not included in the photograph. × 47.

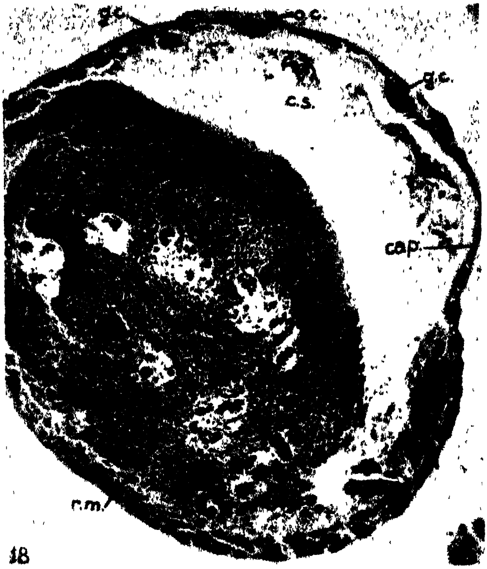
14 This preparation is the same as that for figure 13. The photograph shows the growth center situated in the connective tissue of the old capsule (as shown in fig. 13), a portion of the old capsule, and a portion of the area within the capsule, all slightly magnified to show the character of the tissues. × 76.

18 Section of a parathyroid (R. catesbeiana) fixed in Helly's fluid and Müller's fluid, April 30th, and stained with acid fuchsin and methyl green. This photograph shows a large regenerated mass of parenchyma within the old capsule and also other centers of growth of later origin. × 60.

19 Section from a parathyroid (R. catesbeiana) fixed in Zenker's fluid and stained with iron hematoxylin and picrofuchsin. This figure shows a blood vessel in cross-section, and illustrates the endothelial nuclei protruding into the lumen of the vessel. × 600.

20 Longitudinal section through the center of a parathyroid (R. catesbeiana) fixed in Carnoy's fluid, February 27th, and stained with haemalum and eosin. This photograph illustrates the degree of vascularity which may be encountered in the parathyroid of this species. × 76.

Abbreviations are the same as those listed under plate 3.



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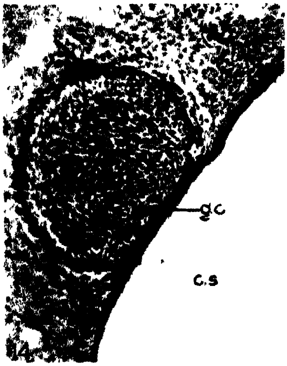

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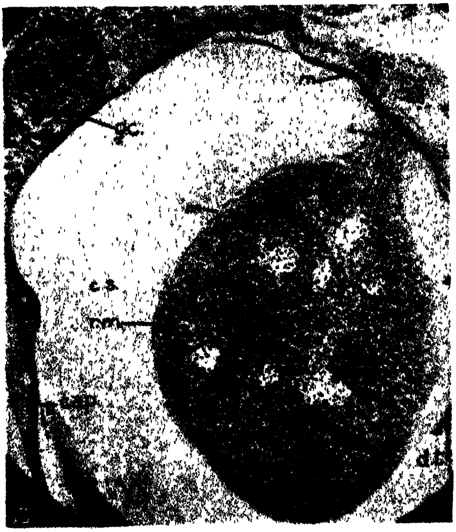

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THE INFLUENCE OF THYROID DEFICIENCY ON THE STRUCTURE AND EPINEPHRIN CONTENT OF THE ADRENAL GLAND OF THE ALBINO RAT (MUS NORVEGICUS ALBINUS)

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THREE HELIOTYPE PLATES (FIVE FIGURES)

AUTHOR'S ABSTRACT

It was found that thyroid deficiency in the albino rat (Mus norvegicus albinus) is followed by a trend to increased concentration of epinephrin in the suprarenals. The basis of this lies in the relatively greater retrogression of the cortex as contrasted with the medulla which becomes a relatively greater proportion of the organ as a whole. Confirmatory evidence is found in cell counts per unit area and the part of the disappearance of lipoid from the cortical cells of the suprarenals of thyroidless animals.

CONTENTS

Introduction 45
Acknowledgments 47
Procedures 47
  Chemical technic . 48
  Morphological and histological technic . . . 49
  Cytological technic 50
Results 50
  Chemical studies 53
  Morphological and histological studies . . 54
  Cytological studies 59
  Lipoid content 63
Discussion 65
Summary and conclusion 68
Literature cited 69

INTRODUCTION

It has long been recognized that the organs of internal secretion comprise a system of coordination for the maintenance of the balance of living mechanisms in the animal. They participate in the reactions of the body as a whole and they probably control growth and behavior.

The thyroid gland is generally believed to be a primary functional center of the incretory group, in that it regulates the metabolic rate. Its function either above or below the normal level produces abnormality. It is, however, not the sole factor in this metabolic regulation.

The interrelationship of the thyroparathyroid apparatus with the other organs of internal secretion has been the subject of numerous investigations. These are not free from contradictory statements. This condition probably arose from the fact that different ages and species of animals were used, associated with the differences in environmental factors such as diet, temperature, humidity, season, health, and hereditary constitution.

Recently, Hammett ('23) found that a lack of thyroparathyroid function brings about a certain distinctive change in the weight of the different glands of internal secretion of the albino rat. The adrenals, pancreas, thymus, and other organs of internal secretion not merely failed to grow, but they showed a high degree of loss of weight or devolution. This was more noted in the females than in the males. He concluded that the thyroid gland is essential not only for growth, but also for the maintenance of these organs.

The adrenal gland, considered as a secondary center of the incretory system, seems to serve as a link between the thyroid and the other organs. It is essential to life. A change in the thyroid produces a change in the adrenal.

Concerning this specific thyroid-adrenal relationship a considerable literature is reported by Herring, Marine, Schafer, Vincent, Bayer, and others. The impression is current that the adrenals tend to decrease in size when thyroid secretion is lacking, and to hypertrophy when thyroid is administered in excess. The evidence is not consistently positive. The various observations on the structural changes and adrenalin content of the adrenals of thyroidless animals are conflicting. Some of the discrepancies are explainable, while others need further investigation. Although fact and theory coincide, the reaction of the adrenal to thyroid deficiency needs more detailed study. The present report is a step toward satisfying that need. A complete review of the literature is practically impossible; therefore, the references are confined to the points of immediate interest.

The present study includes a chemical determination of the epinephrin content of the adrenals of thyroidless rats, a morphological study of the relative volume of cortex and medulla, a histological study of the parts of the gland, and cytological studies of the various types of cells as compared with the normal.

ACKNOWLEDGMENTS

The author acknowledges with pleasure his indebtedness to Dr. F. S. Hammett, at whose instigation the work was carried out, for helping during its progress and for performing certain of the operations. To Dr. C. E. McClung, the Director of the Department of Zoölogy, University of Pennsylvania, many thanks are due for the morphological preparations and helpful comments. The Wistar Institute of Anatomy and Biology and The Research Institute of The Lankenau Hospital provided the materials for the work. To the directors of these two institutions I give grateful appreciation. The study was carried on under the tenure of a Fellowship of The China Medical Board of the Rockefeller Foundation. This paper is in partial fulfillment of the requirements of the degree of Doctor of Philosophy in Zoölogy at the University of Pennsylvania.

PROCEDURES

Wistar Institute rats were used in these experiments. They were the descendants of a single pair, and hence had a common inheritance. The conditions of diet and environment were the same for all, tests and controls. The sexes had been kept separate since weaning. Since the thyroid was removed at one hundred days of age, puberty was not an interfering factor. Litter-mate controls were used in all cases.

The part played by parathyroid deficiency in the thyroparathyroidectomized rats has been discussed by Hammett in his series of papers and need not be repeated here. The results will be discussed as sequelae of thyroid deficiency.

Male and female albino rats were thyroparathyroidectomized at 100 days of age and allowed to grow until 150 days old, when they and their litter-mate controls were killed by ether. The body weight of each individual was taken. The adrenal glands were removed, weighed, and immediately subjected to the test procedures to avoid postmortem changes.

Chemical technic

The epinephrin content of the glands was determined by the method of Folin, Cannon, and Denis ('13), modified by Doctor Hammett to suit the material. There were sixteen male rats and sixteen female rats and their litter-mate controls used in this experiment. Both glands of each pair were ground in a centrifuge tube with fine sand and mixed thoroughly with 1.5 cc. of N/10 HCl and 2 cc. of 15 per cent trichloracetic acid. The mixture was allowed to stand for five minutes. It was then diluted with 4.5 cc. of distilled H_2O and centrifuged for five minutes. Five cubic centimeters of the supernatant fluid was pipetted into a 25-cc. volumetric flask while 2, 3, 4, 6, and 8 cc. or milligrams of uric acid in solution were simultaneously made up in 50-cc. volumetric flasks. To each of these was added 1.5 cc. of N/10 HCl and 2 cc. of 15 per cent trichloracetic acid. To each was added 1 cc. of the reagent (sodium phosphotungstic acid), 6 cc. of Na_2CO_3 solution, and the whole allowed to stand for five minutes. They were then diluted to the mark with water, shaken, and colorimetrically compared. That uric-acid solution of nearly the same intensity of color as the unknown was taken as the standard with the colorimeter set at 20. The values of the readings were reduced to epinephrin concentration in gland tissue. All readings were made within two minutes to avoid error due to color loss.

Morphological and histological technic

The method used for the determination of the relative amount of the cortical and medullary tissue of the gland was that of Allen ('19), modified to suit the material. There were seven male rats and eight female rats and their litter-mate controls used in this experiment. The glands were exposed and injected in situ with the fixative through the blood vessel of the gland until they were hard enough for removal. The fat which surrounded the gland was cleaned off, and the pairs of glands were tied in pieces of gauze and fixed with Allen's fluid for one hour at 38°C. They were then washed with 5 per cent alcohol for half an hour and transferred to 10 per cent alcohol for one to two hours. A bit of lithium carbonate was used for removing the excess picric acid. Dehydration was done by the dropping method from 10 to 50 per cent alcohol for two hours. The glands were transferred to a fresh solution of 50 per cent alcohol to which mixtures of equal volumes of anilin oil and 50, 60, and 70 per cent alcohol were added one after the other for two hours. Thereafter, the glands were transferred to pure anilin oil for ten to sixteen hours until they became transparent. Paraffin impregnation was accomplished by subjecting the tissue successively to 10, 25, 50, 60, 70, 80, and 95 per cent of 52° paraffin mixed with the proportionate amount of wintergreen oil for half an hour each. Finally, the glands were transferred into pure 52° paraffin for two to three hours.

Serial sections of 10 μ were mounted, and stained with Delafield's hematoxylin and eosin. Origanum oil was used for clearing in order to avoid excessive shrinkage. The cortex and medulla of each section of the entire gland were projected onto millimeter paper of uniform thickness. The paper volumes of the cortex and medulla were carefully cut out and weighed. From these the percentage of cortex and medulla was calculated.

Cytological technic

The cytological preparations were made especially to study changes in cell size and its relation to the weight of the gland tissue. No attempt was made to study protoplasmic changes nor to determine the value of the connective tissue, or vascular and intercellular spaces. There were four animals used in this phase of the work and two animals for the study of the lipoids, together with their litter-mate controls.

The glands were treated as described. Serial sections of 6 μ were mounted, and stained with iron hematoxylin for the cell count. Ten sections of each gland from the middle of every five-section interval were selected. Four places in each zone of each section in the cortex and at its diagonal axis were subjected to count. The number of cells in a given area was counted by means of an ocular micrometer grating. The area of the zona glomerulosa is equal to only two-fifths of the total area of the micrometer under oil immersion. For the zona fasciculata, a place was fixed just next to the zona glomerulosa and the total area of the micrometer was used. For the zona reticularis, a place was chosen lying close to the medulla. For the medulla, a place was selected free from connective tissue, vascular and intercellular spaces.

For the counting of the chromatic cells, the same material and the same technic were used. Two animals were used. The number of cells was counted in a given area. The percentage in the different zones was calculated.

For the study of the lipoids, the glands were fixed with four parts of 2 per cent osmic acid mixed with fifteen parts of 1 per cent chromic acid and one part of glacial acetic for twenty-four hours. Further treatments were the same as before. No attempt was made to count the granule number, since the difference between test and controls was conspicuous.

RESULTS

Before going on to the recording and discussion of the adrenal reaction to thyroid deficiency, it is well to note the general growth results. The raw data are given in tables 1 and 2. The controls showed good growth during the period of observation, demonstrating the adequacy of the diet. The thyroidless animals in many cases not only failed to grow, but even lost weight. Thus the presence of a thyroid defi-

TABLE 1

The body and adrenal-gland weights and the epinephrin concentration in the adrenals of the female rats used in the chemical studies

SET	RAT NO	BODY WEIGHT		ADRENAL WEIGHT	EPINEPHRIN CONCENTRATION[1]	DIFFERENCE, TESTS FROM CONTROLS
		100 days	150 days	150 days		
		Grams	*Grams*	*Grams*	*Milligrams*	*Per cent*
A	1	150	120	0.0226	4.43	26.6
	3	157	111	0.0218	4.39	25.8
	Control	166	190	0.0517	3.50	
C	1	124	115	0.0228	3.67	31.0
	2	119	96	0.0221	3.77	34.5
	Control	124	143	0.0324	2.80	
D	1	132	146	0.0312	2.46	—26.0
	2	135	145	0.0238	2.93	—11.5
	Control	156	194	0.0437	3.32	
E	2	132	161	0.0302	3.50	—12.0
	3	147	124	0.0232	3.75	— 5.8
	Control	168	210	0.0434	3.97	
F	1	163	210	0.0327	3.14	4.6
	Control	162	198	0.0302	3.00	
J	1	127	144	0.0300	3.46	58.0
	2	138	162	0.0321	5.14	76.2
	3	138	160	0.0345	4.15	42.6
	Control	131	165	0.0369	2.91	
N	1	154	140	0.0222	4.48	—12.1
	Control	133	176	0.0383	5.12	
P	3	125	112	0.0235	4.39	—29.0
	Control	162	187	0.0385	6.16	
Q	1	203	214	0.0243	4.10	8.4
	2	188	193	0.0348	4.04	7.5
	Control	213	213	0.0346	3.78	

[1] Milligrams per gram of gland tissue.

ciency was shown. Adrenal-gland weight in the test animals was slightly greater than in their litter-mate controls in some cases. It was less in twice as many and predominantly in the females. This greater sensitivity in the latter sex is what was found by Hammett ('26) and Riddle, Honeywell, and Spannuth ('24). The absolute terminal values, however, are

TABLE 2

The body and adrenal-gland weights and the epinephrin concentration in the adrenals of the male rats used in the chemical studies

SET	RAT NO.	BODY WEIGHT		ADRENAL WEIGHT	EPINEPHRIN CONCENTRATION[1]	DIFFERENCE, TESTS FROM CONTROLS
		100 days	150 days	150 days		
		Grams	*Grams*	*Grams*	*Milligrams*	*Per cent*
B	3	151	120	0.0197	3.86	20.0
	Control	190	226	0.0231	3.22	
G	1	200	191	0.0176	3.96	27.5
	2	176	218	0.0200	2.69	—13.2
	Control	171	258	0.0175	3.10	
I	1	145	167	0.0174	3.80	— 9.0
	2	170	166	0.0200	4.52	8.3
	3	164	158	0.0167	4.62	10.7
	Control	164	243	0.0201	4.17	
K	1	126	168	0.0189	4.88	15.5
	2	138	202	0.0200	4.82	14.0
	Control	139	225	0.0174	4.22	
M	1	174	230	0.0219	7.79	63.5
	2	179	235	0.0181	5.92	24.2
	Control	167	256	0.0220	4.76	
O	1	199	256	0.0219	6.83	12.7
	2	160	184	0.0215	6.52	7.5
	3	153	157	0.0163	5.52	— 9.0
	Control	188	274	0.0200	6.05	
R	2	152	217	0.0231	4.52	— 8.0
	3	132	153	0.0200	4.67	— 4.5
	Control	137	232	0.0225	4.89	
U	1	187	188	0.0281	3.22	0.6
	Control	160	252	0.0270	3.20	

[1] Milligrams per gram of gland tissue.

of little significance, since there was no record obtainable of the initial weights, and hence a determination of growth after thyroid removal was impossible.

Chemical studies

In general, the total amount of adrenalin ran parallel with the gland weight in any given series. This positive correlation is what is to be expected. It is higher in the females. This is probably due to the greater sensitivity of the organ to thyroid deficiency in this sex. In order to save space, the raw data are not given. They can be calculated back, however, from the gland weights and the amounts of epinephrin per gram tissue, if verification is desired. These latter figures are given in tables 1 and 2.

From the tables it is seen that the concentration of epinephrin tended to increase in the glands of the thyroidless rats as compared with the controls of both sexes. The average increase in ten of the sixteen females was 32 per cent. The decrease in the remaining six was but 16 per cent. In the males eleven showed an increase averaging 18.4 per cent, while five had a lesser concentration by an average of 8.7 per cent.

This result is interesting. Here we have, in general, in the tests smaller glands of less absolute epinephrin content, yet, relative to the actual weight of tissue, a greater percentage of the characteristic chemical compound. Now, epinephrin is found in, and presumably formed by, the medulla. These analytical results suggest that the medulla is more resistant to thyroid deficiency than is the cortex, the part of the gland which does not contain epinephrin in distinctive amounts. It would also seem as if the cortex of the female rats was more affected than that of the males, since the percentage increase in epinephrin was greater. The morphological evidence which is now to be reported sustains these ideas.

Morphological and histological studies

A brief description of the normal morphology of the adrenal glands is an essential prerequisite to the study of the changes induced by thyroid deficiency.

The adrenal of the adult rat is composed of two main portions, the cortex outside and the medulla inside. The cortex takes origin from the mesoblast, while the medulla is ectodermic in origin from the sympathetic nervous system. The medulla is generally believed to be the secretory center of the gland and to have the same morphological, chemical, and physiological characteristics as that of the chromaphil body in other vertebrates. The cortex differs in having no similar characteristics. The time of appearance in embryonic development is also different. The cortex arises earlier than the medulla. There is no apparent phylogenetic relationship between these two components, nor has a physiological association been demonstrated. Occasionally, some brownish pigment granules of epinephrin are found in the inner zone of the cortex where the two components meet. This, no doubt, is what has led Hartman ('23), Löwenthal ('20), and Cramer ('18) to conclude that epinephrin is produced in the cortex and stored in the medulla, and that the cortex and medulla are functionally similar. On the other hand, Elliot ('06) observed an interweaving of the cortex and medulla along the fringed edge where they meet, and Jackson ('19) showed that the cortex and medulla gradually separate from each other with age. My specimens also show a clear sharp separation between the two parts, and sometimes the granules of epinephrin appear in the inner border of the cortex. However, since there is no phylogenetic relation nor chemical or physiological association between cortex and medulla, there is no justification for the belief of a functional identity, or an epinephrin-forming power of the cortex.

With regard to the physiological significance, the statements are more contradictory than definite. On the one hand, many old views claimed that the medulla is more essential to life than the cortex, while, on the other, Biedl ('13), Vincent

('10), Glynn ('12), Zwemer ('24, '25), and others proved that the cortex is absolutely essential. Again, Exner ('20) reported that the adrenal gland as a whole had no vital significance. However, since the tissue exists, it at least has its own specific function in the living mechanism.

Now, in sections stained with hematoxylin and eosin the cortex is red as contrasted with the blue medulla. In the cortex three zones can be distinguished. The outermost zone is the zona glomerulosa, next to it is the zona fasciculata, and the innermost is the zona reticularis. Connective tissue, vascular and intercellular spaces are also formed in the cortex, the more in the inner, the less in the outer, zone. The area of the zona fasciculata is the broadest and that of the zona glomerulosa the narrowest of the three. The medullary portion only shows a mass of cells forming a syncytial network rich in vascular and intercellular spaces and connective tissue.

Turning now to the experiments, a description of the method is in order. The purpose of this phase of the work was to determine the relative reaction of cortex and medulla to thyroid deficiency. This was done not only for its own sake, but also in an endeavor to find an explanation for the chemical differences already reported.

As stated in a preceding paragraph, the organs were fixed, embedded, serially sectioned, and stained according to standard procedures. Then projections of each section of the entire gland were carefully made on cross-section paper, outlining the medulla. The separation, by cutting, of the cortex and medulla as drawn on the paper gave, of course, material for comparison of the relative sizes of the two parts. The actual comparison was made by weighing all the pieces of paper constituting the cortex projections and all the pieces of paper of the medulla, for a single gland. From these the relative volumes of the two parts to the gland as a whole were directly computed.

Paper weights, in a method of this sort, are obviously not indices of relative weights of parts of an organ, since the paper is of uniform thickness and hence of uniform weight per unit area, while the separate parts of the gland are of different specific gravity and hence of different weight per unit section. The values, then, are indices of relative volume only.

The analytical data, together with the beginning and ending body weights of this series of animals, are given in tables 3 and 4, for the male and female rats, respectively.

TABLE 3

The body weights, weights of the paper projections of the whole adrenal glands, the weights of the cortex and medulla separately, and the proportionate values of cortex and medulla in the female albino rats[1]

SET	RAT NO.	BODY WEIGHT		ADRENAL WEIGHT, 150 DAYS			RATIO TO TOTAL GLAND	
				Both glands				
		100 days	150 days	Total	Cortex	Medulla	Cortex	Medulla
		Grams	*Grams*	*Grams*	*Grams*	*Grams*	*Per cent*	*Per cent*
T	1	143	171	174.478	156.857	17.622	90.0	10.1
	2	132	167	245.339	224.977	20.362	91.7	8.3
	Control	154	197	336.946	315.514	21.433	93.64	6.4
Y	1	115	89	136.931	123.367	13.564	90.0	10.0
	3	152	162	303.802	285.510	18.292	94.0	6.0
	Control	167	187	281.448	259.797	21.651	92.4	7.6
Z	1	154	138	219.584	199.522	20.062	90.9	9.1
	2	157	146	272.617	249.519	23.098	91.5	8.5
	3	147	110	181.237	162.194	19.043	89.5	10.5
	Control	152	182	300.637	284.922	15.715	94.8	5.2

[1] The paper weights of tests and controls were obtained under like conditions and simultaneously, to avoid changes due to atmospheric-moisture variations.

The figures for the total adrenal weight, and cortex and medulla weight, are of course those of the combined paper projections.

Of significance is the fact that in this series, as in the one used for the chemical studies, the total adrenal substance in the tests tended to be less than in the controls, and that this difference was more consistent in the females and of greater degree. This finding, by a different method, of a like general directional and sex difference in reaction of the glands to thyroid deficiency serves to confirm the phenomena as real and not accidental.

TABLE 4

The body weights, weights of the paper projections of the whole adrenal glands, the weights of the cortex and medulla separately, and the proportionate values of cortex and medulla. Males

SET	RAT NO.	BODY WEIGHT		ADRENAL WEIGHT, 150 DAYS			RATIO TO TOTAL GLAND	
				Both glands				
		100 days	150 days	Total	Cortex	Medulla	Cortex	Medulla
		Grams	*Grams*	*Grams*	*Grams*	*Grams*	*Per cent*	*Per cent*
S	1	188	163	126.265	111.484	14.781	88.2	11.8
	3	164	153	155.402	137.675	17.727	88.6	11.4
	Control	193	247	233.837	214.274	19.564	91.6	8.4
V	1	166	199	130.850	113.965	16.885	87.1	12.9
	2	152	200	151.102	134.285	16.817	88.9	11.1
	Control	169	234	142.887	127.719	15.168	89.4	10.6
W	1	168	203	199.949	184.242	15.707	92.1	7.9
	2	173	223	170.013	151.555	18.457	89.1	10.9
	3	126	155	213.859	195.335	18.525	91.3	8.7
	Control	158	254	203.402	187.981	15.421	92.4	7.6
X	1	189	172	181.549	163.458	18.091	90.0	10.0
	Control	164	230	162.988	144.049	18.939	88.5	11.5

The adrenal cortex and medulla of the females, when considered separately, were less than those of the controls in a greater number of times and to a greater degree than in the case of the males. This was to have been expected from the gross values. The adrenal cortex of the female thyroidless rats was 36.3 per cent less than in the controls. In the males the cortex was 19.5 per cent less. The same direction of sex difference obtained in the case of the medulla, but less in degree; viz., 21.1 per cent in the females and 12.9 per cent in the males. Hence, since the cortex is the larger part of the gland, it is to be inferred that the tendency of the adrenal of the female to be more sensitive to thyroid deficiency lies in its cortex rather than the medulla. This holds for the smaller glands. Also, since the absolute difference in both sexes was less in the medulla than in the cortex, it would look as if the size reduction were due largely to cortical reaction. This is substantiated by the changes in relative volume of cortex and medulla after thyroid removal, as shown in the tables.

Here it is seen that the percentage of cortex in the gland was quite generally less in the tests than in the controls; while pari passu the percentage of medulla was greater. The average increase in medullary tissue was 59.5 per cent in the female adrenal and 23.2 in that of the male.

These facts demonstrate that it is the cortex of the glands which reacts most unfavorably to the disturbances produced by thyroid removal. From which the explanations of the finer sex differences in consistency and degree of reaction grossly are to be gleaned, as well as the explanation of the relative increase in epinephrin content. For it is well known and attested to by my data that the adrenals of the female albino rat are larger than those of the male. Also, that the percentage of cortex is greater. Hence it is clear that the sex difference in size is largely a matter of cortex, which, being the more sensitive part with respect to thyroid deficiency, conditions the greater regression in total size found in this sex.

It is true that my results in the projection studies differ from those of Jackson ('19) in the sex difference in cortical and medullary proportions. But Jackson did not make projections of every single section of the glands he studied, and without this an accurate relationship cannot be determined.

It is probable that the cortical loss is what determines the regression of weight reported by Hammett ('26) in his thyroidless rats, although medullary shrinkage probably participated to a slight extent.

The increase in the relative proportion of the gland comprised by the medulla is adequate explanation of the trend to increase in epinephrin concentration found in the chemical studies. Though the quantitative studies are not alike for mass and epinephrin changes, the direction of difference is the same. The lack of quantitative agreement is explicable on the basis of biological variability, lack of sufficient numbers of animals, and probable differences in functional activity of the glands in question. The noteworthy thing is not the lack of quantitative agreement, but the amazing conformity in direction of difference. When one considers the multiplicity of possible variables, the gross correspondence is of added significance.

Cytological studies

A brief description of the cells of the adrenal in the normal rat is the natural introduction to the recital of the changes induced by thyroid deficiency.

The cells of the medulla are larger than those of the cortex. The order of cells on the size basis in the cortex is: fasciculata, reticularis, and glomerulosa. The size of the nucleus of the glomerulosa is least. The cell arrangement of the glomerulosa is the most compact, with few or practically no intercellular spaces save some blood vessels and sinuses. That of the zona fasciculata is comparatively loose, with a median amount of connective tissue and vascular and intercellular spaces; while that of the zona reticularis is spongy in structure and holds a large amount of intercellular spaces and vascular and connective tissue.

There are two different kinds of cells in the cortex. One is the chromatic cell which stains with iron hematoxylin to show a uniformly black-colored nucleus. When stained with Delafield's hematoxylin and eosin, the nuclei are pinkish, somewhat like those of young cells. They are different from the other cells. They are generally found in the zonae glomerulosa and reticularis more than in the fasciculata.

The other kind of cell is the predominant one. Its nucleus when stained with iron hematoxylin shows granules stained as black dots. No cell division was found at this age.

The lipoid granules show in the cortex, especially in the zona fasciculata, less in the glomerulosa, and very few or practically none in the outer margin of the reticularis, when fixed with the osmic-acid mixture. This fixative gives a preparation which shows numerous black granules under the oil immersion.

Medullary cells usually appear in the cortical tissue. In most cases they form one or two or even more strands which extend from the periphery of the cortex inward to connect with the medulla proper. In some cases they scatter here and there in the cortex. This fact Jackson ('19) has reported in young rats. He stated that this strand may not become confluent with the center until a week after birth. According to Poll ('99) and others, the time required for this varies with various animals. The specimens used in my experiments were quite old, however. The glands subjected to observation were 150 days old, yet the medullary strands still persisted. It is unknown whether this condition persists throughout life. There is no satisfactory information on the subject. Kampmeier ('27) found that giant cells appeared in the cortical tissue of the newborn human adrenals. Perhaps it is the same with young rats.

Turning now to the experimental data, I shall first consider the cell count. This was made as described earlier and for the purpose of comparing cell-number change with adrenal-volume change. As originally planned it was intended to study cell volume. But this was impossible, because of the great variability. It is impossible to interpret a change of cell number in a given area of the adrenal as a change in cell volume, since the gland tissue, both the cortex and the medulla, contains a large amount of connective tissue, and vascular and intercellular spaces. On the other hand, it is possible to say that the change in cell number in a given area is due to the change in volume of the gland tissue as a whole. Consequently, no attempt was made to measure the volume of the cells; but only to estimate the volume change in the glandular tissue as indicated by the change in cell number.

Using the technic described in an earlier section, the results recorded in table 5 were obtained. Comparing the tests with their litter-mate controls, it is seen that there was uniformly a greater number of cells per unit area in the former. The average increase was 32 per cent in the zona fasciculata, 26 per cent in the zona glomerulosa, and 38 per cent in the zona reticularis. The increase in the medulla was 26 per cent. Only a few glands were analyzed in this manner, since the reaction was found to be so consistent, not only in the series examined, but also with the results of the chemical and morphological studies.

These results support the preceding ones on different animals in demonstrating a shrinkage of the adrenal in conditions of thyroid deficiency. They show that the cortical shrinkage is greater than that of the medulla. The data as a whole are amazingly consistent.

TABLE 5

The numbers of cells in unit areas of the two adrenals of thyroidless rats and their litter-mate controls

SET	RAT NO.	ZONA CORTICALIS						MEDULLA	
		Glomerulosa		Fasciculata		Reticularis			
		Number	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*
C'	1	77	42	115	40	168	60	83	36
	2	75	39	96	17	126	20	69	13
	Control	54		82		105		61	
D'	1	65	6	123	63	143	43	109	46
	2	74	20	83	9	129	30	81	8
	Control	62		76		100		75	

Visual representation of the change in corticomedullary ratio is given in figures 1 and 2, of a control and test adrenal respectively, drawn to the same scale.

Turning to the study of the chromatic cells, the material and technic have already been described. Briefly, it was found that the cell number of the test animals, on the average, was 33.6 in the zona glomerulosa, 45.6 in the fasciculata, and 147.1 in the reticularis. In the controls there was an average of 26.9 in the glomerulosa, 30.2 in the fasciculata, and 68.5 in the reticularis. The distribution is represented in figure 3. The average increase in the tests as compared with the controls was 20.6 per cent in the glomerulosa, 50.7 in the fasciculata, and 112.7 per cent in the reticularis. This finding, although quantitatively different, is in the same direction with the type of result of the previous observations, particularly in that the inner zone is the highest.

This relatively greater increase, however, cannot be designated as the chief factor in determining the volume change of the cortex, because, beyond the shrinkage of the tissue volume, there are other factors which may indeed change the physicochemical properties of the nucleus of certain cells after thyroid removal. Nor can it be designated as an indication of degeneration of the cells. On the other hand, it is possible that the degree of shrinkage on the inner zone is highest because the chromatic cell is, as it were, a young type of cell, which is more sensitive to disturbance.

Concerning the changes in the relative volume of cortex and medulla, many early workers noted that the ratio changes with age from birth to puberty. Jackson ('19) held the same view and suggested that it is due to erosion, absorption, or degeneration from the corticomedullary border by virtue of medullary expansion. The rate of change was said to depend upon the rate of erosion from the corticomedullary border and the rate of regeneration on the outer zone of the cortex. No such assumption can be dragged in to explain the reaction to thyroid deficiency. In the first place, adrenal growth from 100 to 150 days is slow in the albino rat, the gland being essentially mature at this time. In the second place, no evidence of cell division was obtained in any of my specimens. And in the third place, the paper projections show the medulla of the tests to be less in volume than that of the controls. Hence no positive evidence is had in these directions.

Another old view with regard to this change was held by Gottschua ('83) and Minot ('97). They believed that the permanent medulla was a transformation product of the cortex. Hence the cortex decreases in volume while that of the medulla increases. Embryologically, the two components have two distinct and separate origins, and they have no association in phylogenetic history at all. It is impossible to believe that one is capable of being transformed into the other.

But one explanation is justified from my consistent data, and that is that the relative increase in medulla volume is due to the relatively greater sensitivity of the cortex to thyroid deficiency, with its resultant greater degree of growth retardation, or degree of growth regression. As will be shown, this is probably determined by the lipoid reaction which characterizes the cortex.

The lipoid content

Concerning the changes in the cortical lipoid content produced by thyroid deficiency, I do not mean a change in kind, but a change in amount in relation to the retrogressive change in the gland as a whole. The lipoid of the adrenal is not simple; it is a complex mixture consisting of lecithin and cholesterol and their derivatives. They vary in quality and quantity in various animals. They are also found in all the organs of the body. They are alterable by experiment. Although thyroid secretion is not a specific factor, yet it is one of the factors which is capable of changing the lipoids in the body tissue, including that of the adrenal gland. In this the concentration is predominantly in the cortex.

Turning to my experiments, the lipoid picture in the adrenal cortex of the normal rat has already been described. In the thyroidless rats the lipoid has entirely disappeared from the cortex, and the cells show quite clear with no black granules in the protoplasm. For comparison, figures 4 and 5 are given. A diffuse darker band, which appears along the border between the fasciculata and glomerulosa, may be regarded as a remnant trace of lipoid.

This disappearance of lipoid from the adrenal cortex is an expression of the metabolic disturbance provided by thyroid deficiency on fat metabolism. Catabolism is greater than anabolism, and a proper storage of lipoid is inhibited. This results in a general decrease of fat content from the body—

which is a matter of common observation. That the reaction extends to the adrenals is shown by my results.

This, then, is probably the chief factor productive of the cortical shrinkage demonstrated in the preceding phases of the study. Now, lipoid is much more concentrated in the cortex than the medulla. Since lipoid is particularly depleted by thyroid deficiency, it is clear that the increase in medulla proportion of total gland is really due to a relatively greater shrinkage of cortex because of its relatively greater lipoid content, which is peculiarly sensitive to the catabolism of thyroid deficiency. And the greater sensitivity of the female is due, similarly, to the greater proportion of cortex.

It would be interesting to speculate on this cortical reaction, especially since Bayer states that the lipoid content of the adrenal cortex increases when cultured in media containing thyroid extract. Is the reaction direct and specific, or is it merely an expression of the general bodily lipoid loss following thyroid removal? I am inclined to favor the latter interpretation. Consistent with it is the fact reported by Elliot and Tuckett ('06), that adrenal lipoid varies with the muscular activity.

Early reports regarding the change in lipoid content of the cortex produced by thyroid deficiency were made by Marinesco and Parhon ('08), Mott ('17), Wegelin ('26), and others, and showed a diminution. Gley ('14), on the other hand, found hypertrophy and lipoid increase. His result perhaps was due to inadequate controls.

The effort to explain the significance of the lipoid content has led to much guessing. Biedl suggested that it must have a great value. Others believe that it represents the active secretion of the cortex and is connected with sexual function. Sajous ('25) believes that it is concerned in thermogenesis, while Cramer ('26) thinks that activity of the gland is conditioned thereby, and inhibition by its disappearance. The statements are more suggestive than conclusive.

In a few of my thyroidectomized rats a trace of thyroid tissue was found at autopsy. In no case, however, was there sufficient regeneration (sic) to prevent the characteristic effects of deficiency from becoming evident.

DISCUSSION

The endocrine organs are factors in the determination of metabolic activity. Other factors are, of course, diet, environment, health, and hereditary constitution. Each organ of the incretory system furnishes a specific function, and the activity of one influences that of others either directly or indirectly. This brings up the question of general interglandular relationship, which, however, is too big to be included here. The discussion will be limited to the experimental data actually concerned. Other factors need not be considered, since the material used in my work was adequately controlled.

Now the active principle of thyroid is thyroxin. Kendall ('18), its discoverer, regards this substance as a catalyzer regulating the fundamental chemical reaction rate of the body. In other words, it is used to maintain the level of the metabolic rate. In the case of thyroid insufficiency this is slowed, and the animal not only fails to maintain its normal growth, but even retrogresses, even though the diet is rich in nutrients. My results are consistent with this theory. The body size became smaller and the hair became rough and dull. The adipose tissue was greatly reduced, especially in the females.

This is of interest in connection with the current ideas with regard to infantilism. Early workers considered this due to thyroid deficiency. On the other hand, Vincent ('10) and Glynn ('12) believed the status is related to adrenal-cortical secretion through a hypothetical relation to the reproductive system. Hammett ('26) noted that a specific weight correlation exists between adrenals and gonads, particularly in the female, but not between the thyroid and the gonads. My data show that thyroid deficiency inhibits growth of the adrenals, particularly that of the cortex. It may be that the inhibition of the cortex is a factor in the production of the condition simulating infantilism.

With regard to the assumed functional relation between thyroid and adrenal, Cramer ('16) believes the two glands a part of the mechanism for the chemical heat regulation of the body. Cramer and McCall ('18) state that thyroparathyroidectomy alone in rats does not produce any severe disturbance of metabolism. Hence the interglandular relationship is one of supplement. Massey ('27) reported that thyroidectomy and adrenalectomy each cause a fall of basal metabolism, but a combination deficiency produces a reaction greater than either alone. Thus each gland is stated to be functionally independent, and one can take the place of the other. Marine and Lenhart ('20) noted that the decrease in metabolic rate after thyroidectomy is gradual. Aub ('22) reported that the action of thyroxin and epinephrin on metabolism is not interdependent; that of the former is manifest in the calorigenic action without the adrenal, that of epinephrin is exhibited when thyroid is lacking. Thus one can take the place of the other in metabolic maintenance. While the whole matter is still uncertain, it does appear as if the adrenal is more close and direct in responding to thyroid activity than the other incretory organs, and it may be subordinately and coordinately a participant in the processes of metabolic regulation and maintenance. My results give no support to the idea that the adrenal takes over the function of the thyroid.

As far as the changes in the adrenals of thyroidectomized animals are concerned, three types of observation appear in the literature. The first of these is the record of changes in weight or size. Bensen ('02) reported no change; Vincent ('25), likewise. Goldburg ('27) claims hypertrophy in all cretin sheep and goats. The weight was the same as the controls, or slightly increased, and with marked increase in medullary tissue and cortical hypertrophy. Beyond, he could find no changes. This observation seems dubious to me. Herring ('20) found that the adrenals lost weight per unit body weight. Hoskins ('10) found no change in thyroidectomized newborn guinea-pigs. But he failed to consider the effect of subsequent long-continued growth as related to age at time of removal. This relationship changes after puberty. Hammett ('23, '26) found definite loss in weight when the thyroid was removed after puberty. No workers, save the latter, used controls to give the size of the organs at the time of thyroid removal.

The second type of observation relates to the structural changes in the adrenals. Hofmeister ('94) reported no change. Biedl ('13) stated that the cortex showed a slight hypertrophy. Coulaud ('22) reported that atrophy of the thyroid is always reflected in the cortex. Cramer and McCall ('18) noted that thyroidectomy in rats and rabbits produced slight histological changes in the cortex. Gley ('14) noted that the thyroidectomized rabbit showed cortical hypertrophy. It was loaded with fat. Marine ('22) believes the changes are largely confined to the cortex. Marinesco and Parhon ('08) recorded the changes in lipoid content of the cortex in the dog. Schafer ('24) states that the cortex is somewhat enlarged. Lorand ('09) found a retrogressive growth of the gland in senile myxedema. Mott ('17) described a marked diminution of lipoid in a case of myxedema. Bayer records hypertrophy in rabbits and a change in the amount of lipoid, especially in the fasciculata. These conflicting reports, both in the field of growth and that of morphology, seem to be due to a lack of appreciation of proper control. My studies, in which such controls were had, show definitely not only a retrogression of cortical substance together with a reduction in lipoid, but also an absolute decrease in medullary tissue, though to less degree.

The third type of observation concerns the change in epinephrin content. Lyon ('23) noted that thyroidectomy lessens the response to epinephrin. Herring ('20) found no change in rabbits and cats. He suggested that parathyroidectomy, not thyroidectomy, is responsible for the loss of epinephrin. But Georgopulos ('12) found no difference in the glands of normal and parathyroidless rabbits.

My results differ from these earlier ones. I cannot help but believe that they are correct, since the morphological and cytological studies made by me on different sets of rats give a change which is consistent with that of the epinephrin.

No reference as to cell count in unit area of the adrenals in conditions of thyroid deficiency is to be found in the literature. The shrinkage indicated in my results is evidence of a lack of nutrients, which is consistent with the theory that thyroid lack conditions a lowered metabolic rate. Quite similar is the fact that tissue or cells cultured in inadequate media grow smaller or atrophy. The several parts of the adrenals show varying degrees of susceptibility in this direction. The cause is unknown.

SUMMARY AND CONCLUSION

The adrenals of male and female albino rats 150 days of age were examined for epinephrin concentration, cortico-medullary-volume changes, and lipoid content. The picture found in animals thyroparathyroidectomized at one hundred days of age was compared with that found in their litter-mate controls. Thyroid deficiency was established by the fact of growth retardation or retrogression. The significant results were as follows:

1. The epinephrin concentration was increased in the adrenals of the thyroidless animals.
2. The relative proportion of medulla was also increased. Three proofs for this were had, all obtained on different sets of animals. The first was by actual volume determination by means of paper projections of serial sections of entire glands. The second was the fact of the increase in total-cell count in unit area, this increase being greater in the cortex. And the third was the fact of the increase of chromatic cells per unit area of cortex.
3. The lipoid of the cortex disappeared.
4. The adrenals of the female were more often affected than those of the male.

These findings, made on separate sets of rats, lead to the following conclusion.

Thyroid deficiency increases catabolism, as shown by the loss in body weight. Body lipoids are particularly utilized. Since the adrenal cortex contains much lipoid, its loss is the probable basis of the glandular loss in weight and volume. The medulla, containing less lipoid, is less affected. Hence it increases in relative volume. The increased proportion of medulla determines the increase in epinephrin per unit weight of gland.

The correctness of the interpretation is sustained not only by the gratifying consistency of all the observations obtained on different sets of rats, but also by the fact that the glands of the female were more affected than those of the male. In the female the adrenal is larger than in the male. The difference is constituted by a greater cortex percentage. Since this is the more sensitive, a relatively more marked reaction would be expected in the gland of greater relative cortical volume, and that is what occurred.

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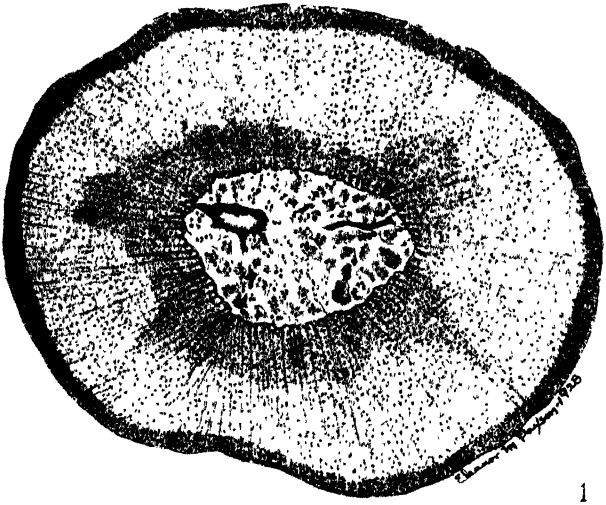
PLATES



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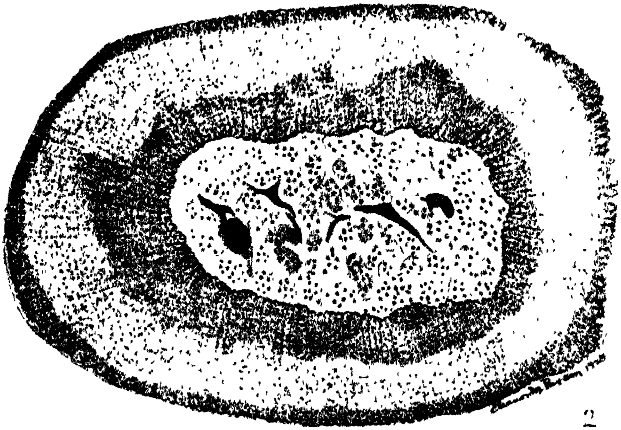

2

PLATE 1

EXPLANATION OF FIGURES

1 and 2 Sections showing the difference in the relative amount of cortex and medulla of the adrenal glands of the control and test rats, respectively. Drawn from the midsection of the gland with approximately same scales.

PLATE 2

EXPLANATION OF FIGURE

3 Section showing the distribution of the chromatic cells in three zones of the adrenal cortex. *1*, zona glomerulosa; *2*, zona fasciculata; *3*, zona reticularis; C_1, the nucleus of predominant cell; C_2, the nucleus of chromatic cell; *M*, medulla.

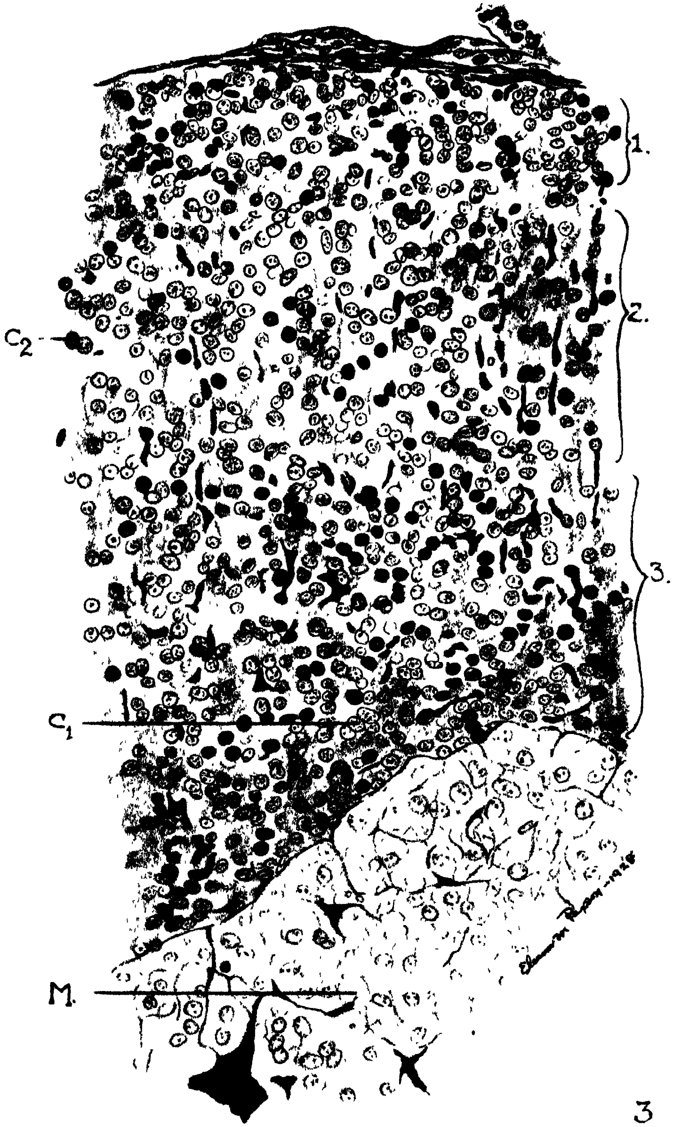

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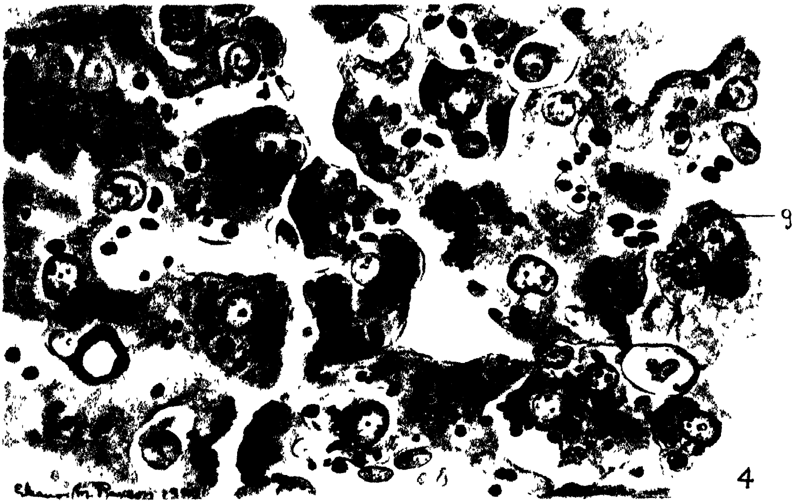
PLATE 3

EXPLANATION OF FIGURES

4 and 5 Sections from the zona fasciculata, showing the difference of lipoid content in the cells of the control and test rats, respectively *a*, the lipoid granules.



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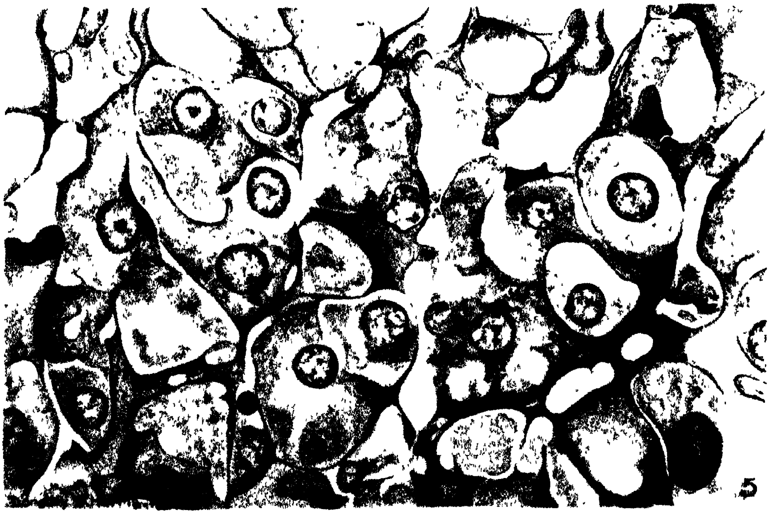

5

THE EMBRYOLOGICAL BASIS OF SIZE INHERITANCE IN THE RABBIT

W. E. CASTLE AND P. W. GREGORY

FIVE PLATES (FORTY-ONE FIGURES)

AUTHORS' ABSTRACT

A comparative study of the embryology of the rabbit in races of large and of small adult size shows a consistently more rapid rate of cell multiplication and of increase in mass in large-race embryos than in those of small race.

This more rapid rate of growth is transmitted by and influenced equally by sperm and egg cell, as is shown by the results of reciprocal crosses.

Rate of differentiation is independent of rate of growth and unaffected by it. Consequently, embryos of the large race have attained greater size than those of the small race at corresponding stages of differentiation.

The fundamental difference in rate of growth is already in evidence in forty-eight-hour embryos and becomes increasingly clear at later stages. Embryos produced by the large race have undergone about one more cell division at forty-eight hours after mating and so are approximately in the thirty-two-cell stage when embryos of the small race are in the sixteen-cell stage.

For several years we have been looking for the thing named in the title of this paper and think that at last we have found it. It consists in a differential rate of cell multiplication in large-race as compared with small-race rabbits, the rate being more rapid in the former. At the same time, differentiation is no more rapid in one race than in the other. Consequently, when the same stage of development has been reached in embryos of both races, the large-race embryo contains more cells (and so larger parts) than a small-race embryo, and so produces a larger individual.

This conclusion was reached by Painter ('28) from a comparative study of twelve-day embryos of the same race which we have employed in this study. It was our purpose to extend to earlier stages the comparative study made by Painter and to learn whether his tentative conclusions held for these earlier stages of development. In particular we were interested to find out whether an endocrine mechanism was involved in producing the differences already observable according to Painter in twelve-day embryos, or whether such differences were already established before the endocrine organs began to function.

We began with the study of embryos as late as could be obtained before attachment to the uterus, which means of the seventh day after mating, or approximately 168 hours. We have studied stages successively earlier back to the forty-eight-hour stage. We shall describe these stages in inverse order to that of our study, in order that the description may agree with the order of the developmental stages.

We are indebted to the Department of Embryology of the Carnegie Institution for assistance, advice, and actual training in the technique of obtaining and studying mammalian embryos. This was made possible through the friendly cooperation of the Director of the Department, Dr. George Streeter, to whom and to the members of his staff our especial thanks are due.

The large and small races of rabbits which form the basis of this study will be described more precisely in another publication. It will suffice for our present purpose to say that the average adult weight of the large race is in females about 5500 grams and in males about 4600 grams. Corresponding adult weights of the small-race rabbits are about 1500 grams for females and 1400 grams for males. It follows that the large race has a body weight more than three times as great as that of the small race. F_1 hybrids have an intermediate adult weight of about 3500 grams for adult females.

Birth weights of large and small races have a similar relation to each other. For the large race the average birth weight of thirteen females and nineteen males was 62.6 grams; for the small race the average birth weight of fourteen individuals, including both sexes, was 35.0 grams. For eight F_1 young borne by small-race mothers and sired by large-race bucks the average birth weight was 55.3 grams; for eighteen F_1 young borne by large-race mothers and sired by small-race bucks, the average was 59.4 grams—a probably insignificant difference. The birth weight of the F_1 young by whichever of the reciprocal crosses it was produced is evidently much closer to that of the large than to that of the small race. This relation holds up to the approach of puberty, when the growth of the hybrids slows up abruptly, while that of the large race goes steadily on. The F_1 hybrids show an acceleration of growth which may perhaps be properly described as heterosis.

Throughout their entire lives the growth curves of these large-race and small-race rabbits are widely separate; the growth curve of the F_1 hybrids is intermediate, but closer to that of the large race up to about 150 days after birth, when it diverges to an intermediate position.

It was our plan to make a comparative study of a series of prenatal stages in both the large (A) and the small (B) races, but our supply of animals proved inadequate for the purpose, as it was necessary to sacrifice the mother in order to obtain timed embryos from her. We have accordingly supplemented our pure race material with embryos obtained from crosses between these same races. One set of embryos came from F_1 mothers back-crossed with bucks of the pure large race. These should be, in the language of animal breeders, three-quarters large-race embryos. We shall refer to them as F-series embryos. Another set of embryos came from females produced by a back-cross of an F_1 doe with a small-race buck. Such females may be considered as three-quarters small-race in constitution. They were now bred to pure small-race bucks to produce embryos seven-eighths small-race, which we shall call D-series embryos. Still another series of embryos was obtained from rabbits of mixed ancestry, which we used chiefly for practice in perfecting our technique, but which may well serve as a control on our observations upon embryos from pedigreed stock. We shall refer to these as C series (control) embryos.

It is known that rabbits ovulate only after copulation, which precedes ovulation by about eight or ten hours. This makes it possible to time embryos accurately within limits of perhaps two to four hours. It has been our practice to breed on the same day rabbits from which we desired to obtain embryos of like age, and then to kill the mothers at exactly the same number of hours after mating. Killing was accomplished by breaking the neck, which results in an instantaneous and painless death, and allows one to operate immediately by opening the body cavity, removing the uteri one at a time and washing them out with tepid normal salt solution (0.75 per cent NaCl). We obtained better results with this than with any of the other salt mixtures which have been recommended for use with mammalian embryos. The embryos were transferred in salt solution to hollow-ground slides, covered with a cover-glass secured with a vaselin seal, and photographed alive with as high a magnification as was available. This, of course, grew less with the increase in absolute size of the embryos. For forty-eight-hour stages we employed a magnification of 180 diameters; for stages of 168 hours we could use a magnification of only 7.5 diameters. After being photographed alive, the embryos were fixed with Bouin's solution and thus preserved for later study. We shall deal in this paper chiefly with the size of the live embryos.

Stage forty-eight hours

We chose as the earliest stage for study one of forty-eight hours after mating, when the egg is in the morula stage and the number of blastomeres is small enough so that the nuclei can be counted with an oil-immersion lens.

We had at this time an insufficient supply of rabbits of the pure large race, and so had to substitute F_1 females, which we mated to bucks of the pure large race. The embryos thus produced would be three-quarters large in their genetic properties as regards size inheritance.

We used four F_1 does in obtaining these three-quarters large embryos, and they gave us a good (F) series of thirty eggs in cleavage stages which were photographed alive in normal salt solution at a magnification of 180 diameters.

Three of these photographs are reproduced in figures 1 to 3. Measurements made upon the negatives show that the over-all diameters of the eggs to the outer edge of the albumen vary from 39 to 57 mm. with the mode on 50 mm., and an average for the thirty eggs of 49.87 mm. The diameter of these same eggs without the albumen, that is, measured to the outer edge of the zona pellucida, varies closely and symmetrically about 31 mm., with an average of 31.13 mm.

For comparison with the foregoing series, we obtained eggs from four does of the pure small race (B) also killed forty-eight hours after mating. They yielded, as expected, a smaller number of eggs, the total being fifteen, or just half the number secured from the F_1 does. The yields per individual were 2, 4, 4, and 5, respectively. The corresponding numbers for the F_1 does were 2, 8, 10, and 10.

The eggs of the small-race (B) rabbits were photographed at the same magnification, 180 diameters, as had been used in the F series. Their over-all diameters range from 46 to 54 mm., average 47.4—about 2 mm. less than the average of the F-series eggs, which was 49.8. The diameter to the outer edge of the zona is nearly the same as in the F-series eggs, being only ½ mm. less, or 30.6 instead of 31.1 as in the F series. For this difference, however, the four eggs of one female (which are shown in figs. 5 to 8) are solely responsible. In both series of eggs the modal diameter is 31 mm. and, except for the peculiar eggs of one individual, the range of variation is exactly the same, closely symmetrical about 31, as shown in table 1.

TABLE 1

Egg diameter (× 180) to outer edge of zona, in millimeters

	28	*29*	*30*	*31*	*32*	*33*
F series embryos,		1	8	10	8	3
B-series embryos (including B 71),	3	1	3	5	1	2
B-series embryos (excluding B 71),			3	5	1	2

We conclude that, so far as egg size is concerned, there is no racial distinction discoverable at the forty-eight-hour stage. This is in agreement with the observations of Painter

('28) upon the unsegmented egg. The eggs of B 71 are slightly smaller than average size as an individual peculiarity. The albuminous envelope of the egg is a secretion of the oviduct and naturally varies more than the diameter of the zona, but even in this measurement it is doubtful whether there is a significant racial difference manifested in the F as compared with the B series.

The case is very different when we come to consider the rate of development of eggs in the F and in the B series, as evidenced by the number of blastomeres formed at forty-eight hours after mating.

In the B series most of the eggs have just reached or are approaching the sixteen-cell stage (thirteen to sixteen cells), with two eggs retarded in the four-cell and six-cell stages, respectively, and one advanced to the thirty-two-cell stage. In the F series most of the eggs are passing from the sixteen-cell to the thirty-two-cell stage, which three of them have attained. None of the thirty eggs of the F series is retarded below the fourteen-cell stage. The average number of cells in an embryo of the B series is 14.0; for the F series it is 21.75. Also more cells are in process of division in the F than in the B embryos. If we count twice each cell in process of division, the number of cells in a B embryo is 14.5 and in an F embryo, 22.8. It is thus obvious that the rate of cleavage is faster in eggs of the F series than in those of the B series, and that the former have at forty-eight hours after mating divided about one time more than the latter, so as to reach the thirty-two-cell stage about the same time as the B eggs reach the sixteen-cell stage. The detailed facts as to blastomere number are summarized in table 2.

Stage one hundred hours

The next developmental stage for which we have comparative data is that of one hundred hours after mating. The living eggs of this stage were photographed at a magnification of 50 diameters and some of them are shown in figures 9 and 10. A doe of the pure large race, A 145, mated to a large-race buck furnished a set of twelve embryos, which we may compare with the seven-eighths small-race embryos furnished by four D does mated with pure small-race bucks. The D does were produced by a back-cross of F_1 does with small-race bucks, and were thus 'three-quarters small-race' animals. Their eggs fertilized by small-race sperm should give rise to 'seven-eighths small-race' embryos. Actually, the eggs of the large-race doe are in a less advanced stage of development than those of the D does, which probably means that they were comparatively late in being fertilized and so starting development. This cannot be proved beyond question, because at present we have only the one set of A embryos of this age. This stage is here described because it is instructive concerning the embryological changes which are in progress at this period. Figure 9 shows the twelve embryos obtained from A 145. It will be observed that the albuminous envelope is being rapidly absorbed as the blastodermic vesicle grows and that the embryonic thickening of the vesicle can be seen excentrically placed in several of the pictures.

TABLE 2

Number of blastomeres in forty-eight-hour embryos of small race (B) and in three-quarters large-race embryos (F)

MOTHER	FREQUENCIES OF BLASTOMERE NUMBERS																			
	4	6	11	13	14	15	16	17	18	19	20	22	23	24	26	27	29	31	32	
B 448							1												1	
B 71		1		1		1	1													
B 253	1			1	1		1													
B 439			1				2	1			1									
Total B	1	1	1	2	1	1	5	1			1								1	Av. = 14.00
F 247													1						1	
F 272								1							2	1		1	2	
F 273							1	1	1	2	1	1	1			1	1			
F 339					3	1	2				2			1						
Total F					3	1	3	2	1	2	3	1	2	1	2	2	1	1	3	Av. = 21.75

In some of the pictures the albuminous envelope is relatively thick, while in others it is nearly gone. As the vesicle increases in diameter the thickness of the albuminous layer grows less. Doubtless the albumen passes into the vesicle in some form or other.

While the albumen is being absorbed, the egg as a whole is increasing in size, which probably means that it is absorbing water or other fluid substances. The average diameter of these twelve eggs over all is 22.6 mm. at a magnification of 50 diameters. The forty-eight-hour eggs at the same magnification would have a diameter over all of less than 14 mm. To the outer edge of the zona, which is rapidly thinning out and vanishing, the eggs of A 145 have an average diameter of 16.3 mm. Eggs of the forty-eight-hour stage in both the F and the B series have a modal diameter to the edge of the zona of 31 mm. at a magnification of 180 diameters, which equals 8.6 mm. at a magnification of 50 diameters. Hence it is clear that between the forty-eight-hour and the one-hundred-hour stages the diameter of the egg, exclusive of the albumen, has just about doubled, while the diameter to the edge of the albuminous envelope has increased something over 50 per cent. Soon the blastodermic vesicle will expand so as to extend beyond the original position of the albumen, which will persist only as a thin membrane surrounding the blastodermic vesicle.

The expansion of the blastodermic vesicle evidently occurs concomitantly with the increase of the over-all diameter of the egg, as is shown by arranging in order of size the measurements of the egg minus the albumen, and in a parallel column the measurements of the egg plus albumen, made on these twelve eggs.

Diameter of blastodermic vesicle	*Diameter over all*	*Diameter of blastodermic vesicle*	*Diameter over all*
8.7	20.7	17.5	21.5
10.7	20.7	17.5	23
15	21	19.5	23
15	22.7	20	25.5
16	22	22	25
16.5	22		
17	24.5	Average, 16.3	22.6

The vesicle evidently grows at the expense of the overlying albumen, since the difference between the two sets of measurements becomes progressively less. It also grows at the expense of the surrounding medium, since the diameter over all steadily increases. As it grows, the albuminous layer becomes thinner and thinner and persists as a delicate membrane only.

From the four D mothers were obtained 1, 4, 4, and 5 embryos, respectively—a total of 14. All these eggs seem to have developed further than the eggs of A 145, already discussed, and to be in very similar stages of development as compared with each other (fig. 10). We conclude, therefore, that they, rather than the eggs of A 145, represent the normal for the one-hundred-hour stage and that A 145 was for some reason retarded. In the D eggs albuminous layer and zona are more reduced than in the eggs of A 145 and the egg as a whole is larger. The measurements made on the negatives are as follows:

Mother	*Diameter of blastodermic vesicle*	*Diameter over all*
D 6,	24.5	26.5
D 21,	22	29
	25	31
	28.5	32
	32.5	35.5
D 101,	24	27
	30	31.5
	33	34.5
	35	36
D 262,	28	34
	38	40.5
	39	40.5
	39.7	41.5
	41	42
Average,	31.4	34.4

Comparing these measurements with those of the eggs obtained from A 145, we see that these have a diameter of the blastodermic vesicle nearly twice as great and a diameter over all about one-half larger, but the thickness of albuminous layer and zona is much less. It is clear, therefore, that the D eggs have progressed further in development, and with their measurements those of the A 145 eggs are not comparable.

Stage 116 hours

For the study of this stage we have nine pure large-race embryos from ♀ A 34, five three-quarters large-race embryos from ♀ F 322, five seven-eighths small-race embryos from ♀ D 73, and as a control five embryos from unpedigreed common stock borne by ♀ C 404.

During the sixteen hours which have elapsed since the one-hundred-hour stage the blastocyst has grown considerably in total diameter; zona and albuminous layers are now extremely thin and delicate and closely applied to the surface of the blastocyst, which has a perfectly spherical shape. In figures 11 to 13 certain of the embryos are shown as photographed alive at a magnification of 20 diameters. Over-all measurements made on the negatives are as follows:

Mother	*Diameter of blastocyst*
A 34 (pure large),	14
	14
	15
	16.5
	18
	18
	20.5
	22.5
	23
Average,	17.9
F 322 (¾ large embryos),	12
	15
	15
	16.2
	18
Average,	15.5

Mother	*Diameter of blastocyst*
D 73 (⅞ small race embryos),	15
	15.7
	17
	18
	19.7
Average,	17.1
C 404 (common stock),	15.4
	16.8
	20.0
	21.3
Average,	18.4

No significant differences are observable between the size of embryos in the races studied at this stage. Although the largest blastocysts occur in the pure large (A) group, the average size of the blastocysts is no greater for this than for the C group and scarcely greater than for the D (seven-eighths small-race) group. The average (at 20 diameters' magnification) is close to 18 mm. in both the A and the D groups. This corresponds to a diameter of 45 mm. at magnification 50, at which the one-hundred-hour blastocysts of the D series averaged 34.4 mm. Consequently, there has been an increase in the intervening sixteen hours from about 35- to about 45-mm. diameter.

Stage 144 hours

For the study of this stage we have one litter of pure large-race embryos, two litters of pure small-race embryos, and five litters from unpedigreed common stock (C) of medium size. In figures 14 to 16 some of the embryos are shown as photographed alive in salt solution at a magnification of 20 diameters. The solutions used when some of these pictures were made were faulty in composition, causing the blastocyst proper to shrink away from the overlying zona. But we believe that the latter was not affected and base on it all measurements made of this stage. The blastocyst is now becoming slightly oval in shape and its diameter as recorded is the mean of the greatest diameter and one at right angles to it in the picture. The measurements are as follows:

Mother		*Average diameter*	
A 267 (pure large),	11 blastocysts,	47.8	(range, 41.5–56.7)
B 57 (pure small),	2 blastocysts,	33.0	
B 437 (pure small),	4 blastocysts,	40.5	(range, 38.2–45)
C (five litters),	22 blastocysts,	45.3	(range, 32–58.5)

The large-race (A) blastocysts are distinctly larger than those of the small-race (B), but not clearly larger than those of unselected stock at this stage. In fixed and stained specimens of this stage, the embryonic area appears darker and rounded in outline, covering between a third and a half of the diameter of the blastocyst.

Stage 168 (167 to 172) hours

The blastocyst at this stage has increased greatly in size and has become more elongated or oval in shape. The embryonic area is becoming pear-shaped and may be distinctly seen even in unstained specimens (figs. 17 to 28). For comparison as to the size of the blastocysts, we have litters from two A mothers, three B mothers, one F, three D's, and two C's. The living eggs were photographed at 15 diameters' magnification and show the following dimensions:

Mother	*Number of embryos*	*Hours*	*Average diameter*	*Range*
A 49 (large),	4	168	75.3	58.2–81.5
A 87 (large),	4	167	84.7	83 –86
F 117 (¾ large),	4	168	83.5	80.5–86.5
D 150 (⅞ small),	4	168	67.9	62.2–72
D 249,	3	169	73.0	66.7–76.7
D 327,	7	168	64.8	58 –73
B 444 (small),	2	172	52.2	38 –66.5
B 447,	3	168	48.4	27.5–71
B 454,	3	167	45.6	34.5–59
C 100 (common stock),	3	168	60.4	55.2–65.5
C 113,	1	167	79.5	

At this stage, while the blastocysts still lie unattached in the uterus, there is clearly evident a distinct difference in size, according to ancestry. Those with a larger proportion of large-race ancestors (A, F) are clearly larger in all their measurements than those in which small-race ancestry predominates (B, D). A similar difference as to number of blastomeres, but not as to egg size, was indicated at the forty-eight-hour stage. Both differences may be ascribed to a common cause—*a more rapid rate of cell multiplication* in the large race than in the small race. This produces a larger number of blastomeres in a given time after fertilization, and this in turn doubtless results in a bigger blastodermic vesicle and an embryonic disk composed of more cells.

Whatever the genetic mechanism of size inheritance is, it apparently operates primarily through a differential rate of cell division in the fertilized egg cell. This rate, we know from the results of reciprocal crosses, is influenced by the spermatozoon as well as by the egg.

There seems to be no reason to think that size inheritance in rabbits is controlled by an endocrine mechanism, either stimulating or inhibiting growth. Dr. R. C. Robb, working in cooperation with us and on these same races of rabbits, has failed to find any evidence of endocrine control. Moreover, in the light of the facts set forth in this paper, an adequate explanation of differences in racial size is found in the differential rate of cell division in zygotes of large-race and of small-race ancestry.

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NOTE. In assembling the figures for reproduction a small part of the photographic print was left outside the limits of the albuminous layer of the egg. As reproduced, this is likely to be mistaken for a part of the egg itself. The reader's attention is particularly directed to figures 9 and 10, in which parts of the original print were not trimmed away in the interstices between eggs lying in close contact. Here the true limits of the almost transparent albuminous layer can be seen.

PLATE 1

EXPLANATION OF FIGURES

Forty-eight-hour living embryos. × 180

1 and 2 From ♀F 273.
3 From ♀F 272.
4 From ♀B 439.
5 to 8 From ♀B 71.

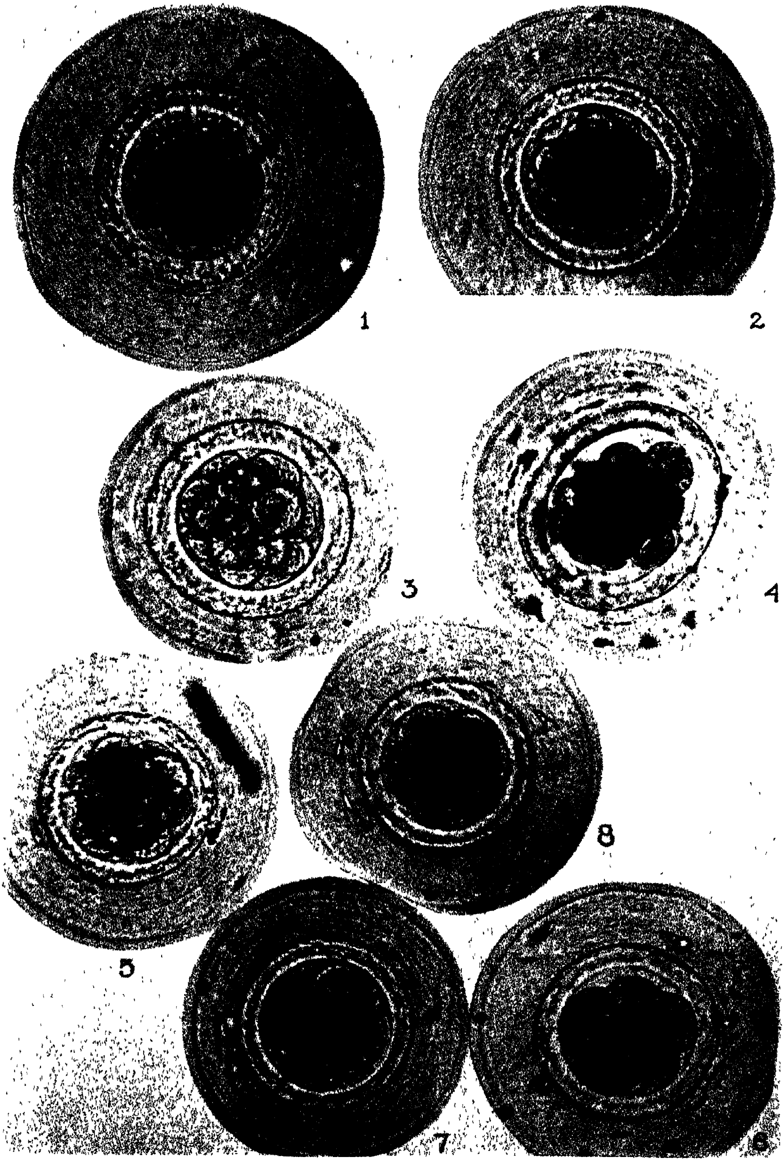
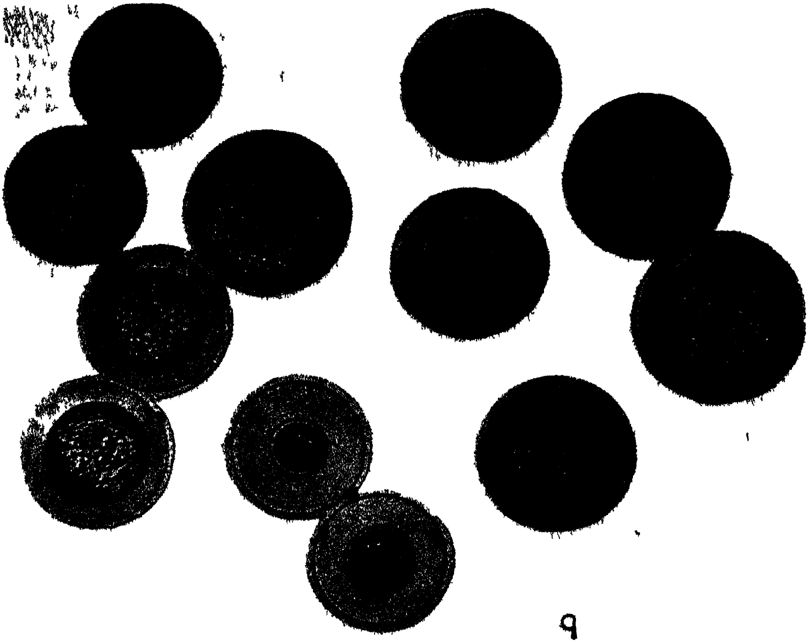

1
2
3
4
5
8
7
6

PLATE 2

EXPLANATION OF FIGURES

One-hundred-hour living embryos. • × 50

9 Twelve embryos from ♀A 145.
10 Five embryos from ♀D 262.



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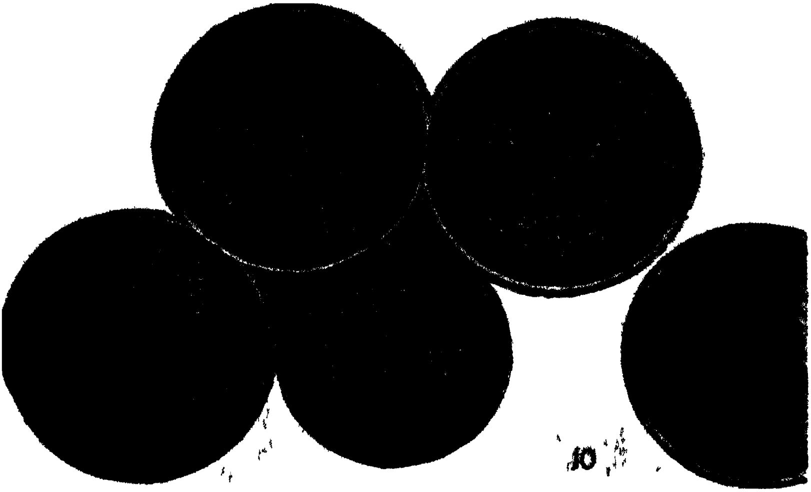

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PLATE 3

EXPLANATION OF FIGURES

116-hour and 144-hour living embryos

11 Nine 116-hour embryos from ♀A 34. × 20.
12 Five 116-hour embryos from ♀F 322. × 20.
13 Five 116-hour embryos from ♀D 73. × 20.
14 Three 144-hour embryos from ♀C 354. × 15.
15 Three 144-hour embryos from ♀B 437. × 15.
16 Three 144-hour embryos from ♀A 267. × 15.

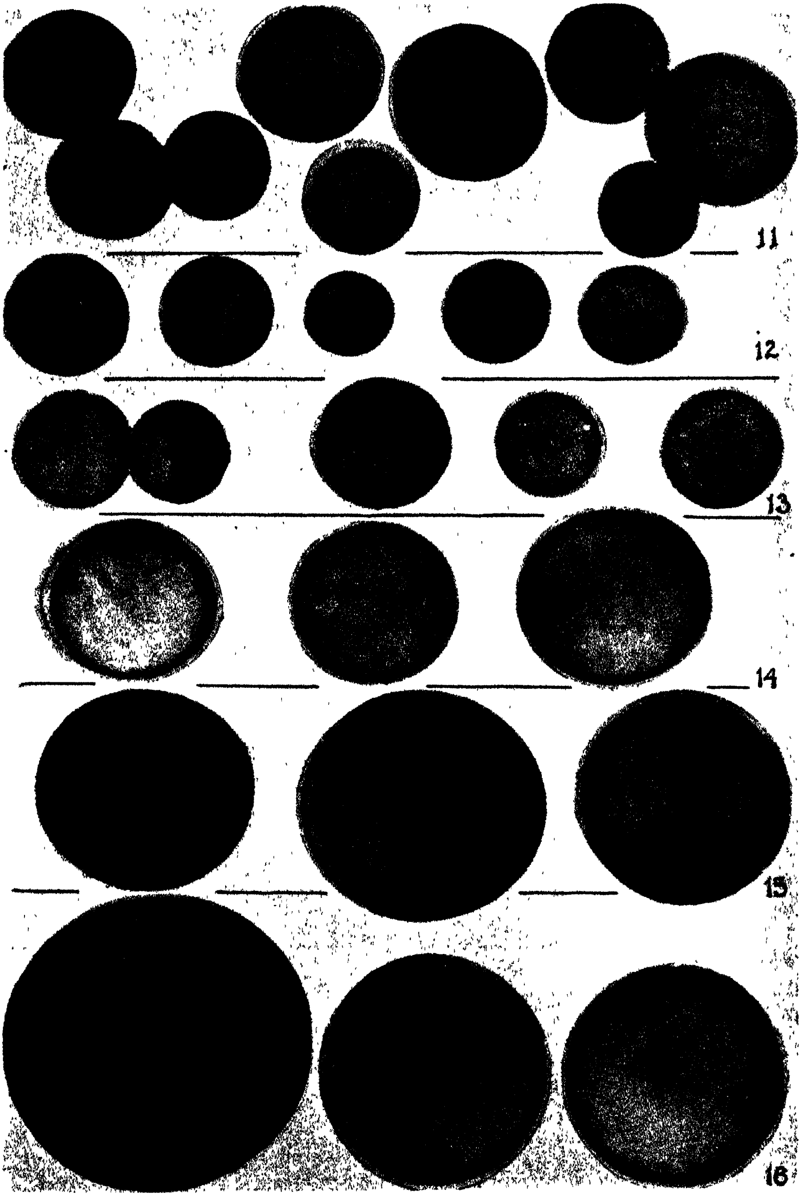
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PLATE 4

EXPLANATION OF FIGURES

17 167-hour blastocyst from ♀A 87. × 7.5.

18 and 19 168-hour living blastocysts from ♀A 49. × 7.5.

20 167-hour blastocyst fixed and stained to show embryonic area, from ♀A 87. × 10.

21 168-hour blastocyst, showing embryonic area, from ♀A 49. × 10.

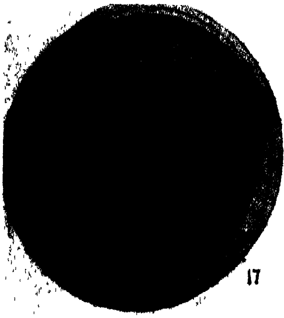
22 Living 168-hour blastocyst from ♀F 117. × 7.5.

23 to 25 Parts of three 168-hour blastocysts from ♀F 117, showing embryonic areas. × 10.

26 Living 168-hour blastocyst from ♀D 150. × 7.5.

27 Living 169-hour blastocyst from ♀D 249. × 7.5.

28 Living 168-hour blastocyst from ♀D 327. × 7.5.



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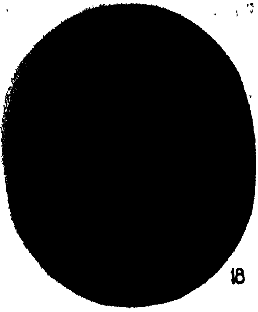

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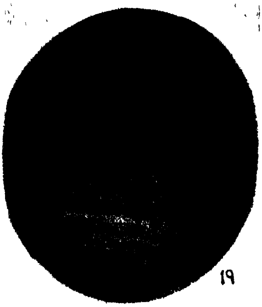

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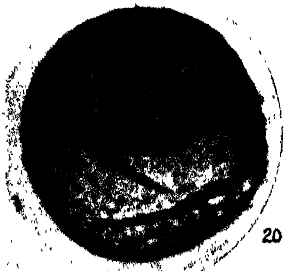

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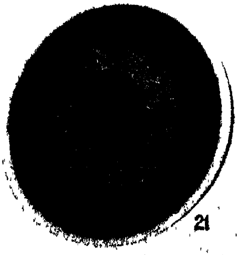

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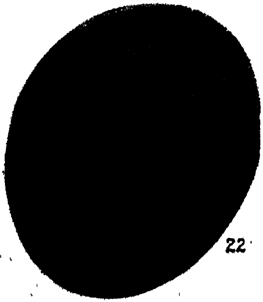

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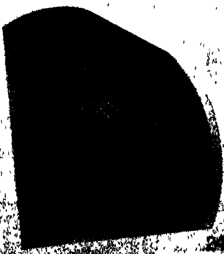

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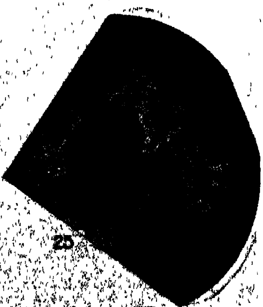

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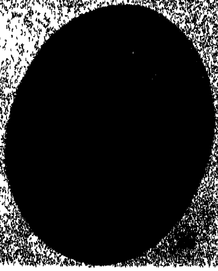

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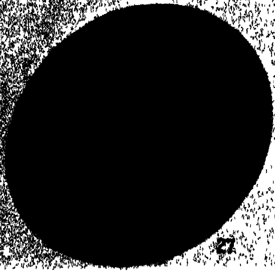

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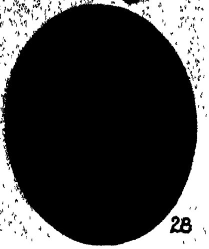

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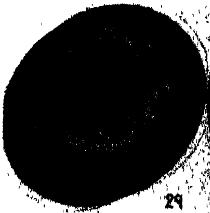
PLATE 5

EXPLANATION OF FIGURES

29 and 30 168-hour blastocysts from ♀D 150. × 10.
31 and 32 168-hour blastocysts from ♀D 327. × 10.
33 Living 172-hour blastocyst from ♀B 444. × 7.5.
34 Living 167 hour blastocyst from ♀B 454. × 7.5.
35 167-hour blastocyst from ♀B 454. × 10.
36 Living 167-hour blastocyst from ♀C 113. × 7.5.
37 and 38 Living 168-hour blastocysts from ♀C 100. × 7.5.
39 167-hour blastocyst from ♀C 113. × 10.
40 and 41 168-hour blastocysts from ♀C 100. × 10.



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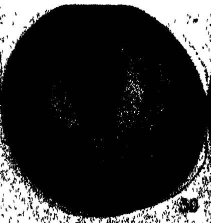

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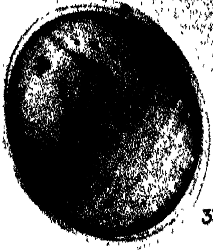

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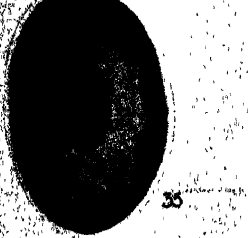

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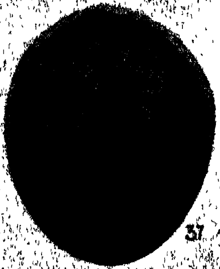

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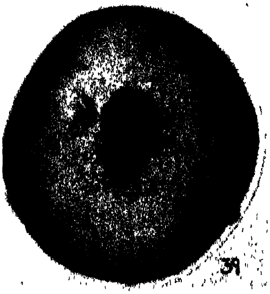

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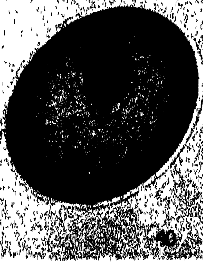

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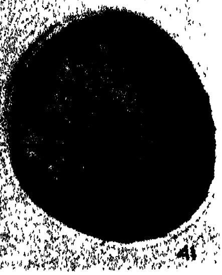

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REACTION OF CERTAIN CYTOPLASMIC INCLUSIONS TO VITAL DYES AND THEIR RELATION TO MITOCHONDRIA AND GOLGI APPARATUS IN THE FLAGELLATE PERANEMA TRICHOPHORUM

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ONE TEXT FIGURE AND ONE PLATE (FOUR FIGURES)

AUTHOR'S ABSTRACT

Two types of cytoplasmic inclusions, differing in reactions to vital dyes and to osmic fixation and impregnation, have been demonstrated in Peranema trichophorum. The mitochondria are rod-like and lie in more or less spiral rows, forming a single layer beneath the periplast. They are stained supravitally with Janus green, but not with neutral red. They may be demonstrated by staining in iron hematoxylin after Mann-Kopsch fixation. They are also blackened in prolonged osmic impregnation, but are bleached readily with hydrogen peroxide.

The small spherical osmiophilic inclusions are scattered in distribution, although sometimes more numerous in the anterior third of the organism. These bodies are stainable supravitally with neutral red, neutral violet, and brilliant cresyl blue. They are denselv blackened in osmic impregnation, and are not bleached in the usual treatment with hydrogen peroxide. They are not, however, demonstrated by Mann-Kopsch fixation and iron hematoxylin. After being stained supravitally with neutral red, these inclusions may be blackened under direct observation by exposure to osmic vapor in hanging-drop preparations.

CONTENTS

Introduction and technique 105
Observations on the living organism 107
Mitochondria 107
Inclusion which resemble Golgi apparatus 109
Discussion 112
Summary 117
Literature cited 117

INTRODUCTION AND TECHNIQUE

The use of vital dyes and methods of osmic fixation and impregnation has shown that, aside from the various cell organelles, there are two general types of cytoplasmic inclusions in Peranema trichophorum. One kind consists of inclusions very similar to mitochondria, while the other type shows properties which resemble those of the Golgi apparatus.[1]

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[1] These observations were reported previously (Hall, '28) in an abstract presented at the 1928 meeting of the American Society of Zoölogists.

In vital staining, clean glass slides are filmed with a solution of the dye in absolute alcohol, according to the method recently used by Dawson ('28). The film is allowed to dry. A drop of the Peranema culture is then placed on the slide, and a cover-slip added. The edges of the cover-slip are sealed with melted vaselin. In such preparations the flagellates will live for three to seven days or more if the concentration of the dye is not great enough to produce toxic effects. The concentration may be controlled not only by varying the dilution of the dye, but also by varying the size of the drop of dye and changing the angle at which the slide is held during the filming process, as pointed out by Dawson ('28). The following dyes were used as 1 per cent solutions in absolute alcohol: alizarin blue S, Congo red, indulin, Meldola blue, methylene blue, neutral violet, Nile blue BB, Nile-blue sulphate, pyronin, and rhodamine. Stock solutions of brilliant cresyl blue and neutral red were diluted 1:15 with absolute alcohol before using, while Janus green B was prepared as a saturated solution in absolute alcohol. A mixture of Janus green and neutral red was prepared with three parts of the stock solution of the former to two parts of dilute neutral-red solution.

Permanent preparations were made by the Mann-Kopsch method of osmic impregnation, and also by fixation in the fluids of Mann-Kopsch and Champy and staining in iron-alum hematoxylin. The method used in handling the flagellates during fixation and impregnation may be mentioned, since it offers certain advantages, in the case of free-living Protozoa, over the procedures cited by Bowen ('28). Material from cultures was concentrated by the centrifuge method (Hall and Powell, '26), and subsequent fixation, osmication, and dehydration were carried out in centrifuge tubes fitted with corks. For free-living flagellates this seems a more satisfactory method than the use of cover-slip smears, as mentioned by Bowen. In the first place, the difficulty of securing satisfactory containers for cover-slips is avoided. Then, the centrifuge method is perhaps more economical in the use of osmic solutions, and there is little or no loss of material such as occurs in the use of cover-slip smears. Furthermore, less time is required for preparation of material in appreciable quantities.

OBSERVATIONS ON THE LIVING ORGANISM

Vaselin-sealed preparations were made for prolonged observation of the organisms under oil immersion. In the living flagellate inclusions of several shapes and sizes are to be observed. The largest constant structures are the spherical or ovoid 'alveoli,' which are probably the characteristic 'vacuoles' seen in material fixed in Schaudinn's fluid. In addition, the cytoplasm often contains several food vacuoles, some of which may be larger than the alveoli. With careful focusing, small spherical refractile bodies are occasionally to be observed scattered throughout the cytoplasm; these are less difficult to detect, however, with dark-field illumination. Just beneath the periplast there is a layer of rod-like or bluntly fusiform structures, the distribution of which seems to follow the spiral surface striations of the body. Other cytoplasmic organelles and the nucleus have been described elsewhere (Hall and Powell, '28), so that the structures to be considered in the following pages are the spindle-shaped bodies immediately beneath the periplast and the small spherical inclusions scattered throughout the cytoplasm.

MITOCHONDRIA

In vital staining with Janus green, the subcuticular spindle-shaped bodies become light bluish green, usually within two or three minutes after the preparation is made. In neutral-red preparations these structures do not take the stain at all, and in mixtures of Janus green and neutral red they are stained only by the Janus green. Furthermore, none of the other vital dyes tried so far has stained these inclusions. In Mann-Kopsch fixation followed by iron-hematoxylin, similar bodies are clearly demonstrated (fig. A). In osmic impregnation blackening of these structures becomes evident

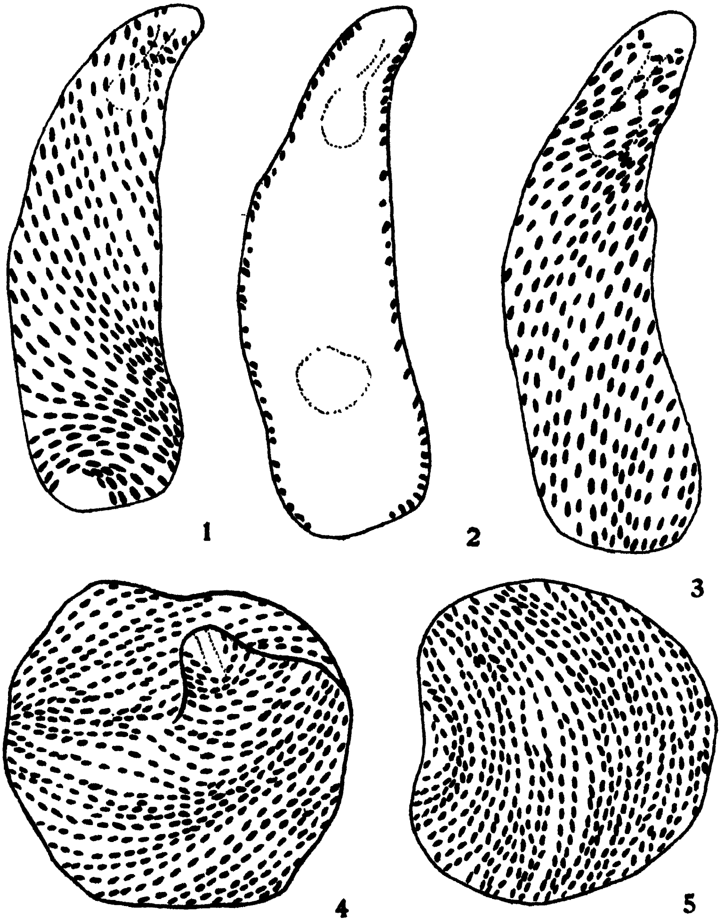

Fig. A Camera-lucida drawings of Peranema trichophorum. Material fixed in Mann-Kopsch and stained with iron-alum hematoxylin. × 2100. 1. Ventral surface view of extended organism, showing mitochondria in rows, forming descending spirals from left to right. 2. Optical section of same organism, showing that distribution of mitochondria is limited to subcuticular layer. 3. Dorsal surface of same organism, showing mitochondria in descending spirals from right to left. 4. Contracted organism, view of upper surface. 5. The same organism, view of lower surface.

by the end of the fifth day, and is accompanied by a distinct darkening of the cytoplasm in general. When such impregnated material is bleached in hydrogen peroxide (diluted 1:10 with absolute alcohol), the rod-like bodies usually become invisible within a few seconds, leaving certain spherical inclusions which are described below. In some specimens bleached for about three minutes, the mitochondria may occasionally be seen with an oil-immersion lens as very light gray, almost shadowy, rods in the characteristic spiral rows. The spherical inclusions, on the other hand, are densely blackened.

Thus the subcuticular rod-like inclusions are stainable vitally with Janus green B, they are demonstrated by the use of an osmic fixative followed by iron-hematoxylin, and they are also bleached differentially, when they are blackened, after osmic impregnation. It is concluded, therefore, that these structures are mitochondria.

The distribution of the mitochondria apparently follows the spiral striations of the surface previously noted in Peranema (Hall and Powell, '28). On the dorsal surface the rows of mitochondria descend spirally from right to left (fig. A, *3*); on the ventral surface, from left to right (fig. A, *1*). As seen in optical sections (fig. A, *2*) of the organism, the mitochondria appear to lie close to the periplast, or in what might be considered the ectoplasmic layer of cytoplasm. It is to be noted that mitochondria are absent in a small area at the posterior end of the body (fig. A, *1*, *2*). On the basis of certain unpublished observations, this region seems to serve as a 'cytopyge.' In contracted organisms (fig. A, *4*, *5*) the arrangement of the mitochondria in rows is still to be seen, although the spirals are not so evident.

INCLUSIONS WHICH RESEMBLE GOLGI APPARATUS

In organisms stained supravitally with neutral red it was found that small spherical bodies take the dye within a few minutes, usually from two to five, after the preparation is set up. These small neutral-red-stainable inclusions are similar in size, shape, and distribution to the small globules, or granules, seen in the living flagellate; and, furthermore, in the flagellates stained with neutral red it has been impossible so far to detect similar inclusions which do not take the dye. It is assumed, therefore, that the granules which are stained with neutral red are the same ones that are present in the normal unstained organism. It is perhaps conceivable that neutral red might cause the appearance of artefacts and at the same time bring about the disappearance of normally preexisting granules of the same size, shape, and distribution as the artefacts, but there seems to be no reason for believing that this actually happens. The usual procedure of making a neutral-red smear and placing the preparation under an oil-immersion lens requires about one minute, and when first observed the granules are stained lightly or not at all. During the next two to five minutes the color gradually deepens as neutral red penetrates the cell. Hence, direct observation supports the conclusion that these preexisting granules of the normal flagellate are the ones stained with neutral red. Furthermore, the appearance of globules stained with neutral red can hardly be considered an indication of a pathological condition, since the normal locomotor activities of the flagellate are continued for a week or more in the average sealed preparation.

These inclusions were not stained by Janus green. It was possible, therefore, by using a mixture of Janus green and neutral red, to stain the mitochondria with the former and the small globules with neutral red at the same time. In such preparations the mitochondria were usually stained shortly before the globules had taken up a visible amount of neutral red. There is no doubt, therefore, that these two types of inclusions differ somewhat in physicochemical nature.

In neutral-red preparations the small globules, or granules, become rather deeply stained and appear sharply defined. In addition, a few of the cytoplasmic vacuoles (alveoli?) may occasionally show a very faint pink color. The food vacuoles are stained also, but rather deeply, the color varying with the pH of the contents. Among the other vital dyes tried, brilliant cresyl blue and neutral violet seem to be the only ones which will stain granules similar to those demonstrated by neutral red. In both cases the granules stained are similar in size and distribution to the ones stained with neutral red, and it is assumed, therefore, that they are of the same type.

In Mann-Kopsch and iron-hematoxylin preparations the neutral-red-stainable inclusions were never found blackened. On the other hand, in material impregnated with osmic (Mann-Kopsch method) and bleached for varying lengths of time (two to forty-five minutes) in hydrogen peroxide (diluted 1:10 with absolute alcohol), small bodies similar to the neutral-red-stainable inclusions are always blackened. In most cases, however, a varying number of vacuoles (alveoli?) show peripheral blackening; in some specimens (fig. 2) relatively few are impregnated, while in others (fig. 1) blackened vacuoles are numerous. In prolonged bleaching in hydrogen peroxide (forty-five minutes or more) the small inclusions are not altered, but remain sharply defined and dense black in color. The vacuole 'rings,' on the other hand, show a tendency to fade out (fig. 3), and in some cases they are bleached almost entirely (fig. 4). It should be pointed out that, in contrast to the observations of Nassonov ('24, '25) on flagellates and ciliates, blackening of the contractile vacuole in Peranema has never been observed.

The osmiophilic granules vary slightly in size in osmicated preparations (figs. 1 to 4) and also in material stained supravitally with neutral red. The granules are usually scattered throughout the cytoplasm, but in many specimens they are somewhat more numerous in the anterior third of the body (figs. 1, 3). The granules are commonly seen in pairs or in groups of three or more, and occasionally (fig. 1) there is a definite linear arrangement of some of the granules, which may or may not appear to be joined by strands of lightly impregnated substance. Fusion of the granules into distinct rods has not been observed.

In order to observe the direct effect of osmic vapor upon the neutral-red-stainable globules, cover-slips were filmed with the dye and a drop of culture placed on top of the film. The drop was first examined, organisms up, to observe the penetration of the neutral red, and then the cover-slip was inverted over a welled slide containing a few drops of 2 per cent osmic solution (Covell and Scott, '28; Dawson, '28). The edges of the cover-slip were sealed with melted vaselin. The flagellates were killed at once, and within two or three minutes the cytoplasm in general became faint pink in color. The neutral-red globules, however, became brighter in color—more of a scarlet—than before, the change being due, apparently, to a drop of pH. After ten minutes, the neutral-red granules showed a peculiar muddy-red color. At the end of approximately twenty minutes, the red had partly faded out and the granules had become light 'brownish-gray' in color. After two hours, the granules were slightly darker. At the end of about twenty hours, the granules were distinctly darkened to a medium shade of gray, while the cytoplasmic background had become light brown in color. In addition, some of the vacuoles showed a faint peripheral 'darkening.' After seventy-two hours, the osmiophilic globules were dark gray to black in color, and in some specimens the cytoplasm was definitely brown. After five days, the entire organism was darkened to such an extent that it was impossible to distinguish very many separate osmiophilic inclusions. During the first three days there was no indication that the mitochondria were affected by the osmic, whereas the neutral-red-stainable globules were distinctly osmiophilic. The walls of the vacuoles, likewise, were not darkened at first, and both mitochondria and vacuoles began to react only when the cytoplasm in general became distinctly darkened.

DISCUSSION

The mitochondria of Penanema trichophorum are rod-like structures which lie in more or less spiral rows, forming a single layer near the surface of the body. These inclusions were observed by Hall and Powell ('28) in Altmann-Regaud preparations, but they were not sharply defined in the material examined and their shape, size, and distribution were not determined accurately. The use of Janus green B has revealed no additional structures which might be interpreted as mitochondria. The mitochondria in this flagellate appear, therefore, to be limited to the subcuticular layer of cytoplasm —a type of distribution which seems to be unusual in the Protozoa. The orientation of the mitochondria in Peranema is likewise rather striking. In elongated flagellates the long axes of the mitochondria tend to parallel the apparent spiral striations of the surface; they rarely lie in transverse planes, except near the posterior end of the body.

This peculiar orientation of the mitochondria in Peranema trichophorum is somewhat similar to that described by Horning ('27) in Paramecium and Nyctotherus cordiformis. In Paramecium the rod-like mitochondria lie almost parallel to the long axis of the animal, forming a number of longitudinal rows, while in Nyctotherus similar mitochondria lie in transverse rows. Horning suggests that in these ciliates the intervening myonemes cause the segregation of the mitochondria into rows. In Paramecium the myonemes are so near one another that the mitochondria are forced to lie end to end. In Nyctotherus the myonemes are farther apart, "thereby allowing the rod-like mitochondria to lie side by side in a position transverse to the axis of the cell." Whether such factors explain the distribution of mitochondria in Peranema is unknown.

The shape of the mitochondria in Peranema fails to support Causey's ('26) hypothesis regarding the correlation of shape with functions of mitochondria in euglenoid flagellates. Causey has concluded that mitochondria are always associated with metabolic activities of the organism, that rod-shaped mitochondria when present take part in anabolic processes, and that, "regardless of the way the food materials are obtained, and regardless of the type of anabolic activity, the catabolic activities in organisms are of the same fundamental nature, and under such conditions the spherical mitochondria may be demonstrated.'' If Causey's hypothesis is generally applicable to the euglenoid flagellates, spherical mitochondria should always be present in vegetative Peranema trichophorum. So far, however, it has been impossible to find any definitely spherical mitochondria in this flagellate. Without denying the obvious probability that mitochondria are concerned in metabolic activities, it seems unwise at present to attempt to apply the criterion of shape as an index to function of mitochondria in euglenoid flagellates.

As compared with the mitochondria in Peranema, the osmiophilic neutral-red-stainable inclusions show certain distinct differences. The mitochondria are elongated and often somewhat fusiform, while the latter are spherical. The mitochondria are arranged in rows, which tend to be spiral, just beneath the periplast, while the spherical bodies are scattered irregularly throughout the cytoplasm. The mitochondria are stainable vitally with Janus green but not with neutral red, while the reverse is true for the spherical inclusions. The mitochondria are demonstrated by fixation in the fluid of Mann-Kopsch and staining in iron-alum hematoxylin, while the latter do not appear to be blackened at all in such preparations. And finally, the spherical bodies remain densely blackened after impregnation with osmic (Mann-Kopsch method) followed by bleaching in hydrogen peroxide, while the mitochondria do not. Since these spherical inclusions seem to satisfy the conventional requirements for identification of the Golgi apparatus, it is apparent that they are very similar to, if not identical with, the Golgi elements.

The Golgi apparatus of Protozoa[2] may assume various forms in different groups. In the Zoomastigoda, Grassé ('26) concludes that the parabasal body of various parasitic species is homologous with the Golgi apparatus of metazoan cells. In the Phytomastigoda, Grassé has homologized the stigma of

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[2] No attempt is made to review the literature on Golgi apparatus in Protozoa, since adequate surveys are to be found in the papers of Bowen ('28), Grassé ('26), Hirschler ('27), Joyet-Lavergne ('25), King ('27).

Euglena with the Golgi apparatus, while Nassonov ('24, '25) concludes that in Chilomonas, as well as in several ciliates, the contractile vacuole represents the Golgi apparatus. The 'Golgi apparatus' of Peranema trichophorum is thus unlike that described for other species of flagellates.

Golgi elements[3] closely resembling the inclusions found in Peranema have been described (King and Gatenby, '23; Joyet-Lavergne, '23, '25) as discrete rods and granules, often scattered throughout the cytoplasm, in various species of Sporozoa (Coccidia and Gregarinida). And Joyet-Lavergne ('26) has shown that, in some cases at least, these discrete Golgi elements may be stained vitally with neutral red. In a more recent paper Cowdry and Scott ('28) have demonstrated the same types of inclusions in Plasmodium praecox. Rod-like mitochondria were stained supravitally with Janus green, while small globules were revealed with the use of neutral red. These neutral-red-stainable globules and the structures formed by their fusion were blackened with osmic under direct observation, with the resultant appearance of an 'appareil de Golgi typique.'

Although similar to the observations on Sporozoa (Joyet-Lavergne, '26; Cowdry and Scott, '28), the results of vital staining in Peranema differ from those described by other workers in Phytomastigoda. Grassé ('25), for example, found that neutral red and cresyl blue stained the 'vacuome' of Euglena proxima. Similar results were obtained with the same dyes by Dangeard ('23) in two dinoflagellates (Ceratium, Peridinium). In Peranema, however, the vacuoles are stained very irregularly, if at all. So far, the staining of more than a few vacuoles in any one specimen has never been observed, and the staining is always very faint at best. In most of the preparations examined it has been impossible to detect a staining of the vacuoles. The small granules, or globules, on the other hand, are always deeply stained.

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[3] Chatton and Grassé ('29, Compt. Rend. Soc. Biol., T. 100, pp. 281–285), in a paper received after the present manuscript had gone to press, have demonstrated in the dinoflagellate Polykrikos schwartzi inclusions which are strikingly similar to those reported in Peranema trichophorum (Hall, '28).

The vacuoles, furthermore, show an irregularity in reaction to osmic impregnation which is not to be observed in the small globules. In prolonged osmication the vacuoles are blackened along with the rest of the cytoplasm and the mitochondria, but they show a tendency to bleach in hydrogen peroxide. After prolonged bleaching, in fact, most—if not all—of the vacuoles lose their impregnation, while the osmiophilic granules show no effect. It seems rather doubtful, therefore, that the vacuoles in Peranema should be considered a part of the Golgi apparatus.

The fact should be stressed that in Peranema trichophorum one is dealing with inclusions which are already visible in the living unstained organism. Chlopin ('27) found that in various metazoan cells 'inclusions' not previously visible may be demonstrated with neutral red, and afterward fixed and stained by ordinary histological methods. This led to the suggestion that such structures may be artefacts induced by the use of neutral red, and not normal cell constituents at all. This objection cannot be applied to the inclusions described in Peranema, since the structures which have been demonstrated by vital dyes and by other methods are also to be seen in the living unstained flagellate.

The presence in Peranema of inclusions which may be stained vitally with neutral red and impregnated with osmic is in accord with the findings of various workers on metazoan cells. Covell and Scott ('28), for example, have shown that in nerve cells the neutral-red-stainable granules "exhibit a pronounced affinity for osmic acid." The 'strands' formed by fusion of these neutral-red-stainable elements are said to "bear a striking resemblance to the Golgi apparatus as revealed by treatment with osmic acid." Dawson ('28) found, likewise, that the granules of the 'segregation apparatus' in the amphibian erythrocyte are "readily stained supravitally with neutral red and brilliant cresyl blue," are densely blackened in Golgi preparations, and "may also be blackened in hanging-drop preparations by prolonged exposure to osmic acid." Hibbard ('28) also concludes that, in the egg of Discoglossus pictus, the Golgi elements may be stained supravitally with neutral red.

SUMMARY

Two general types of cytoplasmic inclusions, differing in their reactions to vital dyes and to osmic fixation and impregnation, have been demonstrated in Peranema trichophorum. The mitochondria are rod-like inclusions which lie in more or less spiral rows, forming a single layer just beneath the periplast. They are stained supravitally with Janus green, but not with neutral red, neutral violet, or brilliant cresyl blue. The mitochondria may be demonstrated by fixation with osmic (Mann-Kopsch fixative) and staining with iron-alum hematoxylin. They are also blackened in prolonged osmic impregnation, but are readily bleached with hydrogen peroxide.

The spherical osmiophilic granules, or globules, are scattered somewhat irregularly throughout the cytoplasm, although sometimes more numerous in the anterior part of the flagellate. These inclusions are stainable supravitally with neutral red, neutral violet, and brilliant cresyl blue, but not with the following dyes: Janus green B, alizarin blue S, Congo red, indulin, Meldola blue, methylene blue, Nile blue BB, Nile-blue sulphate, pyronin, rhodamine. They are densely blackened in osmic impregnation and are not bleached in the usual treatment with hydrogen peroxide. They are not, however, demonstrated by Mann-Kopsch fixation followed by iron-alum hematoxylin. After being stained supravitally with neutral red, these inclusions may also be blackened under direct observation by exposure to osmic vapor in hanging-drop preparations.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

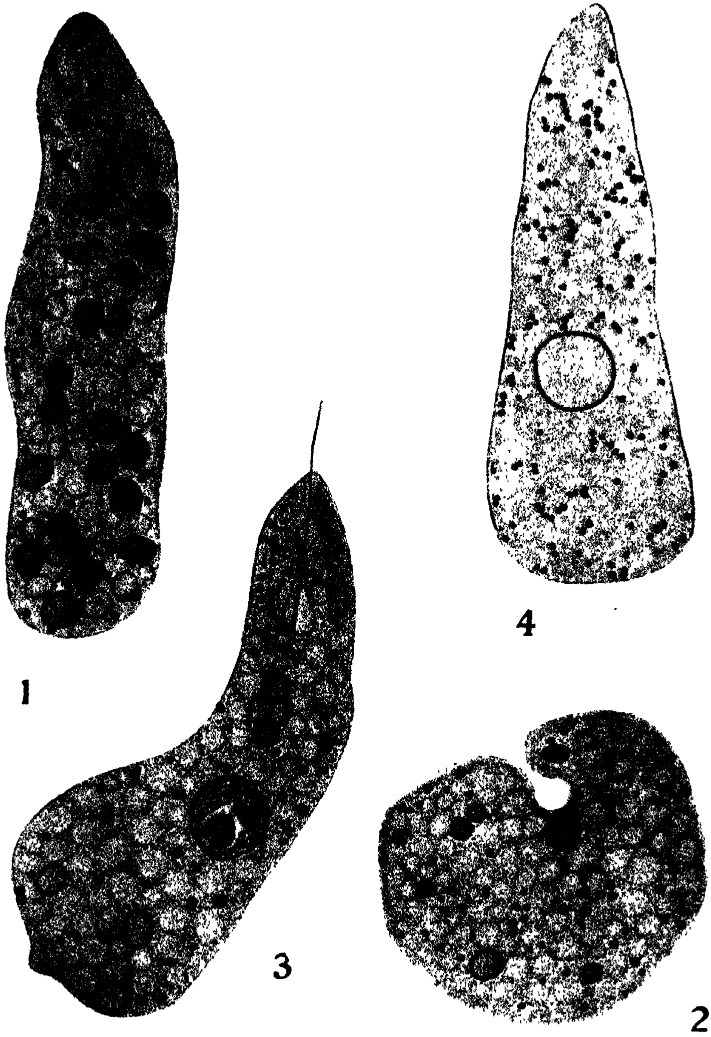
Figures of Peranema trichophorum; material impregnated with osmic (Mann-Kopsch method) and bleached in hydrogen peroxide. × 2100.

1 A flagellate showing numerous vacuoles, blackened in varying intensities, and also the characteristic densely blackened osmiophilic globules. Note aggregation of globules in anterior part of body. Position of gullet indicated diagrammatically. Bleached three minutes.

2 Another specimen from same slide as figure 1. Note relative scarcity of blackened vacuoles.

3 A flagellate from material bleached forty-five minutes. Note that only six vacuoles have retained a slight peripheral 'darkening' which is no more marked than that of the endosomes in the nucleus. The osmiophilic granules show no change, however. Position of gullet and flagellum indicated diagrammatically.

4 Optical section of another flagellate from material bleached forty-five minutes, showing no blackened vacuoles by numerous osmiophilic globules. Position of nucleus indicated diagrammatically; nuclear membrane not blackened in preparation.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GROWTH AND MATURATION IN THE PARTHENOGENETIC AND SEXUAL EGGS OF MOINA MACROCOPA

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THREE HELIOTYPE PLATES (THIRTY-FOUR FIGURES)

AUTHORS' ABSTRACT

From a study of over 1000 mothers, the female chromosome number appears to be 2N = 22, N = 11. The male number has not been exactly determined, but is presumably not haploid. Only one maturation division occurs in the parthenogenetic egg, and the authors have seen only one in the sexual egg. During the growth stages of the eggs, the chromatin is totally obscured by a large amount of deeply staining nucleolar material which exhibits several phases Ultimately, this material is apparently absorbed into the ooplasm. Just before the egg is laid, the ovoid chromosomes, in late prophase or in metaphase, are seen in a germinal vesicle situated always at one side of the egg. The maturation division occurs immediately after egg laying A degenerate body, hitherto undescribed, is noted in the ripe parthenogenetic egg, situated at the pole opposite the germinal vesicle. It is believed to arise by reorganization of nucleolar substance. The body in the sexual egg described by Weismann and Ishikawa ('91) as the Paracopulationzelle is noted and its interpretation by these authors questioned, but, for lack of sufficient evidence, no counter-explanation is offered. The possible relation between experimental sex control and the time of maturation division in the parthenogenetic egg is discussed.

CONTENTS

Introduction 124
Material and methods 124
Observations 128
  General characteristics of the two kinds of egg 128
  Detailed characteristics of each type of egg 128
  Growth stages 130
    For the parthenogenetic egg 130
    For the sexual egg 130
  Characteristics of the growth stages 131
  Maturation 135
    The chromosomes 135
    Reduction 137
Discussion 138
  The nucleolar substance and the unknown body 138
  Weissmann and Ishikawa's Paracopulationzelle 139
  The achromatin 139
  The chromosomes 140
  The possible relation between the chromosome complex and experimental sex control 141
Conclusions 142
Bibliography 142

INTRODUCTION

Numerous workers with Cladocera have found indications that environmental conditions are more or less influential in determining whether the parthenogenetic egg shall develop as a female or as a male. More recently, Banta and Brown's ('23, '29 a, b) evidence has definitely shown that the sex produced by the parthenogenetic eggs of Moina macrocopa is subject to control measures, and that the critical period for this control is (at 20°C.) approximately four hours before the parthenogenetic eggs are laid. It seemed of interest to secure information concerning the possible chromosome mechanism involved and, if such a mechanism were found, to relate the cytological findings with the experimental control of sex.

Little is known of the daphnid chromosomes, and a correlation between sex and chromosomes has not been worked out for any of them. In this study it was hoped that we might be able to analyze the male and female chromosome complexes of Moina macrocopa. While the main object has not been fully accomplished, the number of chromosomes in the female has been determined, as well as the fact that the male number probably is not haploid. The growth of the nucleus with its unusual nucleolar constituent and a cytoplasmic body hitherto unrecorded are described, and Weismann and Ishikawa's interpretation ('91) of their Paracopulationzelle is questioned.

MATERIAL AND METHODS

The first author is responsible for preparation of slides and the cytological findings. The second author supplied the ample living material, timed the stages desired, and furnished the life-history data. The material came from the second author's large stock of Cladocera at the Department of Genetics, Cold Spring Harbor. Upward of 1000 individuals were sectioned. Cladocera are very susceptible to changes in temperature, their physiologic processes becoming very slow at temperatures below 20°C. The time records mentioned in this paper refer, unless otherwise stated, to temperatures of 20° to 23°C.

Reliable criteria for determining the time of any particular stage in the growth process of the eggs, by observation of the living mothers, were found only for the late maturation stages. In both the parthenogenetic and the sexual egg the maturation division occurs within five minutes after egg laying. About five minutes before a clutch of parthenogenetic eggs is laid, the mother molts and discharges from the brood chamber the preceding clutch of young—events easily seen with the naked eye. The ripe eggs may thus be fixed in the mother's ovary immediately after discharge of the young. The corresponding time for the sexual egg was fixed by the act of molting, since these mothers do not have young in the brood chamber. (Sexual-egg production in a given individual of this species may precede, but does not follow, the production of parthenogenetic eggs.) Immediately before laying, the chromosomes of both types of egg are in metaphase or very late prophase.

No means was found of determining in advance when molting would occur, nearer than by three or four hours. Pigmentation in the eyes of embryos in the brood chamber was watched as it developed, but it is practically complete three or four hours before molting. Variation in molting time is so great in mothers of the same age reared under as nearly identical conditions as possible that we were never sure that mothers killed two, three, or four hours after eye pigmentation seemed complete would yield eggs in a particular stage. As prophase stages occur in the latter part of this period, they were difficult to procure. In spite of large numbers of mothers sectioned during this period, there is still a regrettable gap in this part of the story (between figs. 20 and 21 to 24).

For the anaphase and polar-body-formation stages, the parthenogenetic mother must be killed almost immediately after the eggs are passed into the brood chamber. Cleavage in the nucleus may begin in parthenogenetic eggs five minutes after laying, or perhaps earlier at temperatures above 22°C.

For the sexual egg during the period of later ovarian development, size and gross appearance of the eggs in the living animal are the only guides yet found for stages of development which are sufficiently definite to be relied on in fixing material.

The early growth stages of both the parthenogenetic and the sexual egg were fully studied from oogonia up to the time when the chromatin makes its appearance. Several series for parthenogenetic and sexual material were prepared and studied. The production of the sexual egg is readily induced by reducing the food supply of a young female from a few hours after birth. Series for early cleavage stages were also secured. Still another series was prepared for securing parthenogenetic eggs which are to produce males. Under readily obtainable conditions, 80 to 100 per cent of the crowded mothers may be induced to produce female young, in which case the young of the next clutch, due to the influence of crowding, are largely or wholly males (Banta and Brown, in manuscript). Several such mothers were fixed.

Various fixatives were tried. The most satisfactory were two: 1) the combined use of Ohlmacher's fluid and Allen's B-15 (Allen, '16). The mothers were placed whole in the first at room temperature for one-half to one hour, and then in B-15 at a temperature of 38° to 40°C. for an hour. 2) Allen's B-20, which is B-15 plus osmic acid: 1 cc. of a 2 per cent aqueous solution of osmic acid to 50 cc. of B-15. The mothers were placed in this at a temperature of about 38°C. for one hour. Either Ohlmacher's or Carnoy's solution alone tends to swell the chromosomes and causes them to clump to some extent, although both are valuable as general fixatives. The combined fixatives helped rid the yolk of some of its oil globules, but no fixative cleared up all of the deutoplasmic spherules in the sexual egg. Gilson's fluid yielded poor fixation, but it so affected the nucleolar substance that it did not stain deeply with nuclear stains.

As stain, iron hematoxylin was used for the most part. We are greatly indebted to Prof. H. de Winiwarter, of Liége, Belgium, for staining some of our slides with Flemming's triple stain. This was done in his laboratory, where he fortunately had a supply of reliable safranin. We have since tried in the United States various samples of safranin, but have been unable to obtain satisfactory results with any of them. The triple stain is especially desirable for this material, because it differentiates chromatin and various degrees of degeneration somewhat better than does iron hematoxylin. For hematoxylin it was found necessary to mordant about twenty-four hours in a 1 per cent solution of iron alum and then stain in about 1 per cent or 2 per cent aqueous solution of hematoxylin for an equal length of time. By this method, the chromosomes held the stain, while the ooplasmic granules, which crowd about the chromosomes, lost the greater part in differentiation. The granules never became completely decolorized and were a constant source of difficulty in making accurate chromosome counts.

All eggs at or near maturation stages required examination with a 2-mm. oil-immersion objective to reveal the extremely small division figures. A Zeiss 1.5 oil-immersion objective was found helpful for chromosome study, but the best optical combination tried was the new Bausch & Lomb monobjective binocular microscope fitted with their 2-mm. oil-immersion objective and their 12.5 oculars.

We wish to thank Prof. H. de Winiwarter, director of the Institute of Anatomy, Liége, Belgium, for placing his laboratory at the first author's service for several weeks in the summer of 1926. To Miss Louise Buck and to Miss Margaret B. Chambers, as well as to Mlle. Hubin, of Liége, we are indebted for the drawings accompanying this paper. To Bausch & Lomb Optical Company we are indebted for the courtesy of the use of one of their new monobjective binoculars equipped with the lenses as stated above. If this instrument had been available in the earlier study of the chromosomes, much time would have been saved.

OBSERVATIONS

General characteristics of the two kinds of egg

In Moina, as in nearly all Cladocera, there are two types of egg—the parthenogenetic and the sexual. The former may produce either females or males; the latter produces only females. When ripe, they differ markedly in appearance, and in all stages after the deutoplasm begins to be laid down are easily distinguishable in the ovary of the living animal. Throughout the greater part of the growth stages both have the same type of nucleus, in which two very distinct substances are prominent. One of these substances reacts strongly to chromatin stains after most methods of fixation. By previous observers this substance has been called the nucleolus. With the growth of the egg, it increases enormously, breaks up into many irregular bodies of various sizes, finally degenerates, and is absorbed by the ooplasm (figs. 7 to 20). A better designation than nucleolus would seem to be nucleolar substance, since the material forms numerous bodies of various sizes and shapes, and apparently takes no part in the formation of chromosomes, as do some nucleoli.

The other conspicuous nuclear constituent surrounds and interpenetrates the nucleolar substance (figs. 8 to 16, especially 8, 11, and 14). It appears as flocculent material, which stains lightly with cytoplasmic stains. Probably it is a highly fluid gel, its somewhat woolly appearance in sectioned material being due to coagulation.

The chromatin is not discernible during the growth stages of the eggs. Compared with the other substances, it is small in quantity (figs. 1, 20 to 31).

Detailed characteristics of each type of egg

The two types of egg differ in size, in the number produced at a clutch, and in the character of the yolk (figs. 1, 4). The brood chambers also differ. That of the sexual egg develops a conspicuous egg case, the ephippium. Only one sexual egg normally matures in each ovary at a clutch. During growth it absorbs three nurse cells. Other immature eggs may degenerate without being absorbed into the egg cell. Several parthenogenetic eggs (twelve to twenty as an average) are laid at a clutch, the number depending upon the age of the mother, temperature, and nutritive conditions (Banta and Brown, in manuscript). Weismann ('77) reports degeneration of some parthenogenetic egg cells in several Cladocera, but nurse cells as such are not found in this type of egg in Moina macrocopa.

The ooplasm in the ripe parthenogenetic egg is a finely granular substance, scattered through which are rather large vacuoles and oil globules (*Vac.* and *O.gl.*, fig. 1). These begin to appear at an earlier stage (fig. 12). Large lacunae appear in the mature female-producing parthenogenetic egg (*Lac.*, figs. 1, 2). These have not been seen in eggs regarded as male-producing (fig. 3). At present writing, these lacunae are interpreted as artefacts. More study by different methods of fixation is necessary before we may state whether absence of lacunae in the male-producing egg is due to a difference in its ooplasm. The lacunae are elongated in shape and for the most part lie with their longer dimension across the egg, and are most prominent in the zone containing the division nucleus and the degenerate body (*Lac.*, fig. 1). After the egg is laid, they are in about the same position (fig. 2), although during its passage through the oviduct the egg is greatly elongated; in the brood chamber it quickly becomes nearly spherical and then ovoid (figs. 2, 3). The lacunae may mark a weaker region in the ooplasm in preparation for division, as the plane of first cleavage is parallel with the shorter diameter of the egg.

The yolk of the mature sexual egg is composed chiefly of spherules which stain deeply with hematoxylin and other nuclear stains (figs. 4 to 6). About the periphery there is more or less of the finely granular material such as is seen in the parthenogenetic egg (fig. 4).

In the early growth stages no differences are discernible in the two types of egg. The yolk differentiation which distinguishes the sexual egg begins (probably in the neighborhood of thirty hours before the egg is to be laid) at about the stage of nucleus drawn in figure 11, when the nucleolar substance is beginning to break down. At this stage in the sexual egg small yolk spherules are forming in the granular matrix, and the nurse cells show nucleolar degeneration somewhat more advanced than in the egg cell.

Growth stages

Three well-marked stages may be differentiated during growth, the criteria for which are present in both nucleus and cytosome. In the nucleus the changes in the nucleolar substance are the most prominent and will be described in detail below. In the cytosome two changes are readily seen: 1) increase in volume, and 2) changes in structure and substance due to the presence of deutoplasm. In its early stages the cytosome is finely granular, practically homogeneous except for a variable number of small bodies which stain deeply with hematoxylin, one or more of which are usually found alongside the nuclear membrane (figs. 8 to 10).

Successive stages in nuclear development are as follows:

For the parthenogenetic egg

Stage 1. From oogonium to the beginning of the degeneration of the nucleolar substance, and, in the cytosome, the appearance of oil globules (figs. 7 to 11).

Stage 2. From this condition to that marked by a finely granular homogeneous nucleus and a highly vacuolated state of the ooplasm (figs. 12 to 17).

Stage 3 (ripening). The dissolution of the nuclear membrane and the appearance of a spindle and chromatin (figs. 1, 18 to 20). In the cytosome at the end of this stage a 'degenerate body' (*Deg.b.*, fig. 1) appears, and the female-producing egg shows lacunae (figs. 1 and 2).

For the sexual egg

Stage 1. Nuclear stages practically identical with stage 1 of the parthenogenetic egg. In the cytosome, the appearance of clearly differentiated yolk spherules instead of oil globules.

Stage 2. Nuclear changes similar to stage 2 of the parthenogenetic nucleus. In the cytosome, the increased reaction of the yolk spherules to nuclear stains, and the beginning of the absorption of the nurse cells. At about the end of this stage there appears near the periphery of the egg, always close to one of the nurse cells, a darkly staining body (*Un.b.*, figs. 4 and 5), which Weismann and Ishikawa ('91) describe as the Paracopulationzelle, but to which we give the name unknown body.

Stage 3 (ripening). Nuclear: practically as in the parthenogenetic type; cytosomic: no further change in the yolk, but the presence for a brief period of the incompletely digested masses of nurse-cell material (fig. 6).

Characteristics of the growth stages

Stage 1 (figs. 7 to 11). Nuclear phenomena. The oogonia are tiny cells characterized by a small amount of cytoplasm and a well-marked nucleus which contains one to perhaps three or four prominent nucleoli in a matrix of woolly achromatin (fig. 7). The oocytes in their earliest development (fig. 8) differ only in their larger size and, in some cases, by the presence of small ovoid or spherical cytoplasmic bodies which lie near or against the nuclear membrane and stain like chromatin. These bodies persist throughout stage 1 (figs. 7 to 11). The first clutch of eggs is differentiated in the embryo as far as, or slightly further than, the stage shown in figure 9. Growth in size of nucleus is accompanied by an increase in the number and size of distinct bodies formed by the nucleolar substance (figs. 9 to 11). These are of all shapes and sizes, with no constancy in number or specific differentiation. A great deal of time was spent in the attempt to find some one body or portion of the nucleolar substance which might prove to be a chromatin bearer, but we could identify none in either type of egg. Before the nucleus has reached its full size, the bodies begin to lose their staining reaction at the periphery, as shown in figure 11.

In the meantime the second nuclear constituent, the achromatin, has retained the characteristics it possessed in the oogonia, but has increased in quantity. It forms a relatively thick layer between the masses of nucleolar substance and the nuclear membrane, and also penetrates among them (figs. 8 to 14). This achromatin stains lightly with nuclear stains and is differentiated completely by iron alum or by acid after hematoxylin, when it shows its acidophilic character by its positive but weak reaction to cytoplasmic stains (figs. 9 to 12).

Changes in the cytosome. Up to about the end of the first stage the cytosome maintains a finely granular structure, homogeneous except for the variable number of deeply staining small bodies already mentioned. These do not persist beyond the beginning of the next stage, gradually losing their affinity for the stain (figs. 12 to 17). The most striking change in the cytosome is its greatly increased staining reaction near the end of the first stage (fig. 10), just before degeneration appears in the nucleolar substance (fig. 11). At this same period the cytosome begins to increase rapidly, and with its rapid growth and vacuolation the staining reaction weakens, as seen in figure 12.

Stage 2. In stage 2 (figs. 12 to 17) the nucleolar substance seems to break down in two distinctly different ways. 1) The separate bodies shown in figure 11 may either break up into smaller ones (fig. 12), in each of which several pycnotic elements later appear (fig. 15), or, 2) the large bodies shown in figure 11 may vacuolate (figs. 13, 14), after which they and the smaller ones pass to the pycnotic condition (figs. 14, 15). The first of these two methods may be traced successively in figures 11, 12, and 15; the second, in figures 11, 13, and 14. Both may be present in one female, or only the first type described. Whether the second type indicates complete degeneration of such an egg has not been determined.

The next clearly defined condition is shown in figure 16. A mass of granules, uniform in size, occupies nearly the whole nucleus, crowding the acidophilic substance against the membrane. These granules gradually lose their strong reaction to nuclear stains and soon fill the entire nucleus, completely concealing any other nuclear substance which may be present (fig. 17). Intermediate steps between the stages shown in figures 15 and 16 have been found, but are not illustrated in this paper. At this stage we made most careful search for positive evidence of chromatin, but found none.

In the sexual egg the only difference observed in the behavior of the nucleolar substance during this stage is that its masses tend to remain a little more uniform in size and shape (fig. 4), and at the end break down into small aggregates of finely granular nature which take nuclear stains more deeply than the rest of the granular substance (fig. 6). At this stage the nucleus of each type of egg lies close to the egg membrane on the side toward the alimentary canal, opposite to the oogonia and oocytes (figs. 1, 4).

The cytosome increases greatly in quantity, becoming highly vacuolated in the parthenogenetic egg. The vacuoles are filled with a substance which takes cytoplasmic stains faintly. In shape they are spherical or ellipsoidal. In the sexual egg the yolk spheres are prominent and stain as already described (p. 129) and as is well shown in figure 4. The disintegration of the three nurse cells is well along in this stage (fig. 4). In them the nucleolar substance appears in bodies of various size and shape, in contrast with the more nearly uniform bodies seen in the egg nucleus; their cytosome clearly shows degeneration (fig. 4).

Stage 3 (figs. 1, 18 to 20) marks the culmination of growth (by some writers spoken of as ripening). The salient features are the appearance of chromatin in the matrix and the dissolution of the nuclear membrane. The chromatin comes to view inside an ellipsoidal area which, as the nuclear membrane begins to disintegrate, appears clear in the sections and stains slightly pink with eosin (figs. 18, 19). At first this area shows no differentiated structure (fig. 18) or may be composed of fine granules arranged in lines suggesting a spindle (fig. 19). Before the nuclear membrane has completely disappeared, small bodies of irregular shape and of various size are to be discerned against a faintly outlined spindle (fig. 20). They have strong affinity for nuclear stains.

The nucleolar substance seems to have no organized bodies in the first part of this stage, but later there appears at the periphery of the parthenogenetic egg, opposite the intestine, a body which stains deeply with nuclear stains (*Deg.b.*, figs. 1 to 3). This body seems to have escaped notice by previous workers. Until more is known of it, we call it the degenerate body. It consists of a central spherical body surrounded by more or less numerous small spheres varying in size. In their early history they all take nuclear stains (except after Gilson fixation) to about the same degree as the chromosomes.

From its history it would appear that this body arises from reorganized nucleolar substance. It is not present in the growth stages of the egg and first comes to clear view after the germinal vesicle has reached the periphery of the egg. It is then to be seen at the opposite pole of the egg, lying close to the egg membrane. After egg laying, this position is maintained. Steps in its formation have been traced from the dissolution of the nucleus until its formation at the periphery of the egg. After the complete dissolution of the nuclear membrane, it appears in the region of the germinal vesicle. It is then an ill-defined, darkly staining homogeneous mass. In this form it moves to its position at the opposite side of the egg, where it can be detected breaking up into a few masses of irregular shape, out of which the central body and its surrounding asteroid bodies are formed. During the first cleavage it remains in the ooplasm still at the periphery, as in figures 2 and 3. As cleavage proceeds it loses its staining reaction and at about the eight-cell stage or later seems to have become absorbed in the yolk. No evidence has been seen to indicate that it unites with any one of the cleavage nuclei. This migration may perhaps have some relation with the position of the lacunae. The body seems to lie at one pole of the egg. It needs more study. No corresponding body has been seen in the sexual egg.

Just before the nucleus or membrane begins to disintegrate, there appears on the side of the nucleus farther away from the intestine a change in the character of the ooplasm. It takes on the same appearance as that of the granular substance in the nucleus and stains in the same way (figs. 6, 18). After the dissolution of the nuclear membrane, the nuclear contents mingle with the ooplasm (figs. 19, 20). Together they seem to form a matrix for the germinal vesicle, which moves to the edge of the egg next the intestine, where it remains during maturation. In the sexual egg one portion of the matrix retains a more densely staining quality until the metaphase of the first maturation division (fig. 21, *G.s.*), but is lost to view after the egg is laid. The germinal vesicle itself lies in a portion which stains less densely (fig. 21).

One more characteristic of the cytosome in the sexual egg remains to be noted. Just before laying, the material contributed by the nurse cells is still to be seen as a faintly staining granular substance (fig. 6) in different portions of the ooplasm. Traces of this are still to be found during the earliest cleavages, but most of it seems to have been distributed while the egg is passing through the oviduct or as it again becomes ellipsoidal.

Maturation

The chromosomes. Maturation occurs just previous to and after egg laying. It is essentially the same in the two types of egg, except that in the parthenogenetic type there is only one division, and no reduction occurs. In the whole maturation process no spireme has been observed, unless the stage shown in figure 20 may be called a spireme. In what is probably the last part of the prophase, the chromosomes appear as tiny bodies approximately of the same size, spherical or slightly ellipsoidal, which stain deeply with nuclear stains (fig. 22). The ellipsoidal form seems to persist up to late anaphase, when in some cells an elongated form approaching rod shape has been observed at the egg pole (fig. 32). This form has also been found in some anaphase poles at the two-cell cleavage stage. No V's have been seen. The chromosomes in metaphase of the first cleavage nuclei are distinct and may be counted. In later divisions they are very small and difficult to observe.

The first chromosome complexes found were in parthenogenetic eggs just previous to laying. They seemed to indicate a variable number of chromosomes—a puzzling observation. Weismann and Ishikawa ('91) report four chromosomes in Moina paradoxa (now M. macrocopa) and M. rectirostris. Kühn ('08) reports eight in Daphnia pulex. That the number must be higher in our material was at once evident. No basis for a fixed number was at hand until the first maturation anaphase shown in figure 29 had been found. A few favorable early cleavage stages pointed to twenty-two as the diploid number. Then more material was prepared and examined with the best of microscopical conditions. It would now appear that twenty-two may be regarded as the diploid number in the female (figs. 22 to 31).

Not every complex shows this number, but in only one cell not clearly in anaphase have more than twenty-two been found. This one has twenty-four, one chromosome of which may have divided. In figure 22 the largest body is probably two chromosomes, though no clear line of separation was discernible even when destaining was carried to the limit. The presence of one exceptionally large chromosome in a complex, as in this figure, is quite unusual. In general, the chromosomes appear of about the same size. In the parthenogenetic eggs many anaphases have been found. Among the most favorable for counting chromosomes are those shown in figures 30 and 31. The tendency of the polar-body complex to mass quickly is strong. In the cell drawn in figure 30 only sixteen polar-body chromosomes can positively be determined, but the position is somewhat unfavorable. The complex shown in figure 31—an oblique view—is in more favorable position, and twenty-two chromosomes can be determined at each pole. The smaller size of the polar-body chromosomes is shown in figures 30 and 31. No such difference is observable in the first maturation division of the sexual egg (fig. 29). The polar-body chromosomes may also occur in two sizes in the same complex, as shown in figure 31, where six are nearly as large as those at the egg pole and agree in size with the polar-body chromosomes shown in figure 30. Further evidence is needed on polar-body formation.

Some mothers which had been treated so as to cause them to produce more than the usual ratio of males were killed between molting and egg laying, and some immediately after egg laying. None of these yielded division figures in which the number of chromosomes could be positively determined. However, in all cells in which the chromosomes could be seen there was no doubt of sixteen or more. Thus we may be reasonably sure that the male does not carry the haploid number of chromosomes, although we do not yet know whether there is a sex difference in number (Allen, '28). This conclusion is in agreement with genetic evidence in Daphnia longispina (Banta and Wood, '28).

At metaphase of the first maturation spindle, just previous to egg laying, the chromosomes form in a nearly flat plate. The whole process of the formation of the chromosomes and their movement to this position is very rapid, evidently occurring in a few minutes. In material fixed while the egg is in the oviduct, the germinal vesicle is difficult to locate, but as soon as the egg has partly assumed a spheroidal shape in the brood chamber, the vesicle may again be seen with the chromosomes still spread out in a plate (figs. 26 to 28).

Reduction. In the sexual egg, reduction apparently occurs at the first maturation division. Only one sexual egg at anaphase has been seen in which the chromosomes in each group are distinct enough to count accurately (fig. 29). In this case no external polar body was found. In two other cases, in which exact counts cannot be made, the number at the egg pole is obviously too small to be diploid, and neither polar body shows division. The polar bodies have the chromatin massed, as in figure 33. No evidence of a division of a polar body has yet been seen in either type of egg. Early anaphase may be reached in the sexual egg before it is passed into the brood chamber, but we have found no late anaphase or a polar body in such eggs while still in the ovary. The spindles usually lie at right angles to the periphery of the egg, as shown in figures 1, 25, and 31, but a few have been seen at an oblique angle.

DISCUSSION

The nucleolar substance and the unknown body

The prominence of the nucleolar substance and its behavior make it of peculiar interest. However, in this paper no comparison of it with more or less similar substances in other forms besides Cladocera will be undertaken, and, in the Cladocera, only with Daphnia pulex and its pseudosexual form. It needs microbiochemical study. We agree with Kühn ('08) that it is not chromatin "obgleich sie sich mit gewissen Kernfarben ähnlich färbt" (p. 574). That it functions as nutritive material for the deutoplasm seems clear from its behavior. Its decrease in staining reaction and the disintegration of its formed bodies correlate with the accompanying increase in staining reaction and marked acceleration in growth of the cytosome. It seems to be nearly absorbed into the cytosome by the time the nuclear membrane has completely broken down.

A correlation of considerable interest is the fact that the change in the cytosome in the formation of the sexual type of egg occurs in the early stages of degeneration in the nucleolar substance of the egg and nurse-cell nuclei.

In this connection it is of interest to note the difference in behavior of the nucleoli in Daphnia pulex and a pseudosexual species (unnamed) of Daphnia. In D. pulex (Kühn, '08) this substance maintains itself as one body until it has reached the stage in its growth corresponding to stage 3 of Moina as described in this paper. Then it breaks down in the same manner as it does in M. macrocopa before the chromosomes appear. In the pseudosexual species Schrader reports ('25) that the nucleolus forms a hollow sphere, and for a short period after the appearance of the chromosomes, this sphere and all the rest of the nuclear substance stain very intensively with iron hematoxylin. When this reaction ceases, the nucleolus has disappeared, and the nuclear contents are a flocculent, lightly staining groundwork. "In the midst of this lies the chromosome group in connection with which appear the first spindle fibers (fig. 6). None of the flocculent nuclear substance is included in the space occupied by the spindle . . ." (pp. 6-7).

Weismann and Ishikawa's Paracopulationzelle

In Moina macrocopa this body appears only in the sexual egg. In figure 4 it is labeled the unknown body (*Un.b.*). Its first appearance is at about the time the egg nuclei and nurse cells show definite degeneration. At this time it always lies near one of the nurse-cell nuclei, but within the ooplasm (figs. 4, 5). It persists after the egg is laid. Weismann and Ishikawa ('91) describe it as being extruded from the egg nucleus, passing to the periphery, and finally uniting with one of the cleavage nuclei in the eight-cell stage of the embryo—correcting their former conclusion that it is the sperm. Our first interpretation agreed with these writers' earlier conclusion. We reached this conclusion before seeing Weismann and Ishikawa's paper on the subject. Limited evidence in our material throws some doubt on their last interpretation, but we have no proof that the body is a sperm. It is found in eggs which have been exposed to fertilization. We do not know whether or not it occurs in unfertilized eggs. The sexual egg ultimately degenerates if not fertilized. But we have no means of knowing whether a given egg has been fertilized, even though copulation may have been observed. This body is not present until a stage later than that at which the yolk takes on its distinctive character. Further study is needed either to confirm or to disprove Weismann and Ishikawa's interpretation.

The achromatin

We cannot agree with Kühn's ('08) and Schrader's ('25) interpretation as to the connection of this substance with the chromatin. In Moina macrocopa it seems to have no relation to the chromosomes. Where and how the chromatin is carried during the growth stages of the nucleus we do not know, but think that it is probably in a vesicular state. Evidence for this belief is present in the early cleavage nuclei of the sexual egg, but it will not be presented in this paper. The evidence against the chromatin nature of this substance is its almost total disappearance from view after formalin fixation and its consistent negative reaction to nuclear stains. The variability of its reaction to different fixatives indicates that when alive it is a highly fluid gel. That the chromosome vesicles may be borne in it is entirely conceivable, but that the woolly substance which is our only evidence of its presence is partly or wholly chromatin needs much more satisfactory demonstration.

The chromosomes

Both Kühn ('08) and Schrader ('25) think that the chromatin is either connected with, or arises from, the achromatic portion of the nucleus. The former described the fibers as either splitting or pairing in prophase (p. 551 and fig. 25)—an observation which Schrader states he cannot confirm. Kühn reports that after the prophase stages, when the nucleolus has broken down into a mass of fine particles, the chromosomes appear as rods within a finely granular nuclear matrix (his fig. 37) against a lighter background; that in subsequent stages the rod form persists, and V's are formed in early anaphase; and that in later anaphase the spheroidal form appears (the common form found in M. macrocopa). He reports eight chromosomes as the diploid number.

In the pseudosexual form Schrader finds the chromosomes in the shape of well-defined rods, of which there are about twenty-four in the diploid complex. Weismann and Ishikawa ('91) report four as the diploid number in M. paradoxa (now M. macrocopa). However, some of their figures may be interpreted as showing many more chromosomes than four, especially their figure 24 (M. paradoxa), but the authors do not name the bodies in question.

The possible relation between the chromosome complex and experimental sex control

Experimental results on the environmental sex control of Moina macrocopa clearly indicate that the sex to be developed from parthenogenetic eggs is fixed by treatments effective approximately four hours before the eggs are laid (Banta and Brown, '29 b). Whitney ('14) found that in the rotifer Hydatina senta a still earlier determination of sex is present, the sex of the parthenogenetic offspring in this organism being controlled by environmental influences acting upon the grandmothers. Whitney also reports ('28) that in the rotifer Asplanchna amphora the mature parthenogenetic male-producing eggs contain the haploid number of chromosomes, and that in a few of the parthenogenetic eggs the chromosomes in the maturation stages are markedly larger than in the rest of the eggs, but that no correlation has yet been determined between this difference in chromosome size and sex of the individuals.

In Moina macrocopa the cytologic evidence at hand shows that the germinal vesicle (fig. 20) is probably not formed in the parthenogenetic egg until within the second half of the four-hour period before egg laying—and perhaps later. The great variation in sisters of the same clutch makes it difficult to obtain this time relation more closely. Certain it is that the maturation does not take place until the end of the period. Some time is, of course, required for the influence of the changed environment to become active in the eggs. It may be that the eggs which we interpreted as male-producing were actually female-producing, and that a possibility of a haploid number in the male has not been entirely ruled out, in spite of the correlated genetic evidence in Daphnia (Banta and Wood, '28). At any rate, these last four hours in the history of the parthenogenetic eggs of Moina macrocopa and the early cleavage cells present an attractive field of investigation to both the physiologist and the cytologist. To the latter the extremely small size of the chromosomes and the great variation in degree of development in sisters of the same clutch present serious difficulties.

CONCLUSIONS

1. A peculiar body hitherto unrecorded in Cladocera eggs appears in the parthenogenetic yolk after the nuclear membrane has broken down and the chromosomes have appeared in the division nucleus. This body degenerates in the early cleavage stages. It is tentatively called the degenerate body.
2. In agreement with previous workers, the large amount of nucleolar substance is interpreted as nutritive material, not chromatin.
3. The explanation by Weismann and Ishikawa ('91) for the body named by them the Paracopulationzelle is questioned.
4. The chromosome number in the female of Moina macrocopa is apparently 2N = 22, N = 11. The exact number in the male has not been determined, but presumably it is considerably more than eleven; hence it is not haploid. Consequently, we have no information as to the occurrence or nonoccurrence of a sex-chromosome complex.
5. The cytological conditions at the period of environmental sex control by external environmental factors, about four hours previous to maturation division, remain to be accurately determined. No conclusion has been reached as to a correlation between the environmental sex control and a possible sex-chromosomal mechanism.

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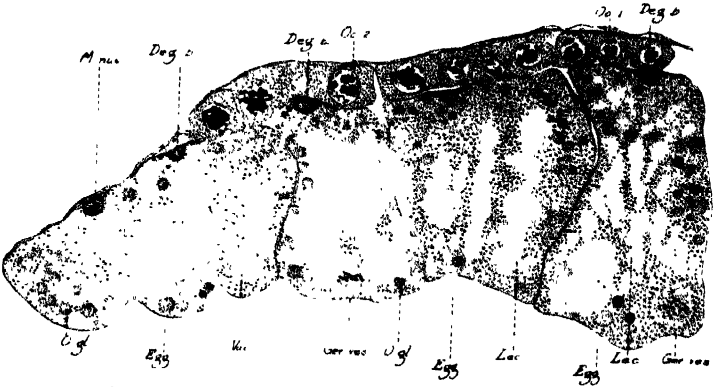
PLATES

The figures are all of sections cut at 7 to 10 μ. The figures of highest magnification were drawn with Zeiss 1.5- or 2 mm. objectives and 12.5 ocular; for the lower magnifications, with a Zeiss 4-mm. apochromatic objective and appropriate oculars. In both cases the outlines were made with the camera lucida and the details afterward filled in. Unless otherwise stated, the killing and fixing fluid was Ohlmacher's and Allen's B 15, and the stain, iron-alum hematoxylin.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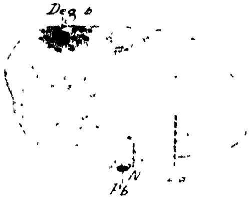
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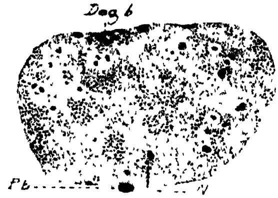
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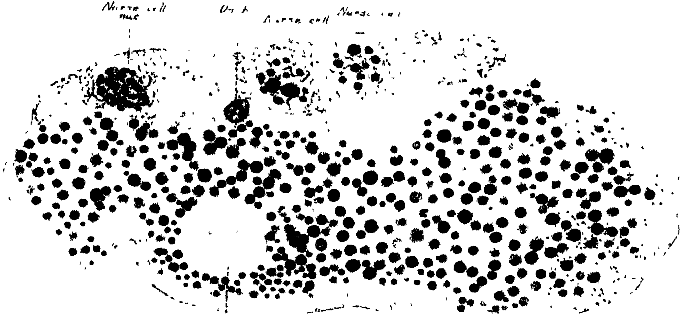
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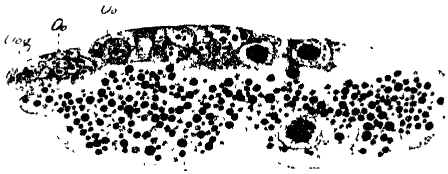
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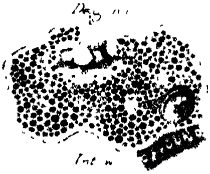
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PLATE 1

EXPLANATION OF FIGURES

Parthenogenetic and sexual eggs, showing their chief characteristics. The lower side of each figure is toward the alimentary canal.

1 Four ripe ovarian parthenogenetic eggs (*Egg.*), just before laying, and some young oocytes (*Oo.1, 2*). Two of the degenerate bodies (*Deg.b.*) and a germinal vesicle (*Ger.ves.*) were introduced from adjoining sections; no one section has been found passing through corresponding levels in so many eggs. The ripe eggs show vacuoles (*Vac.*), oil globules (*O.gl.*), and lacunae (*Lac.*) in the granular ooplasm, and a membrane nucleus (*M.nuc.*). × 450. The chromosomes are slightly exaggerated in size to bring them out at this magnification.

2 A parthenogenetic female-producing egg five minutes after laying, showing the polar body (*P.b.*), cleavage nucleus (*N*), and degenerate body (drawn in optical section) (*Deg.b.*), the characteristic lacunae still extending across the egg chiefly in the central zone. × 450. (Figure 33 was drawn from this egg.)

3 A parthenogenetic presumably male-producing egg killed after being laid (exact time not known), showing the lack of lacunae in ooplasm and some densely staining bodies in the deutoplasmic spherules. It lacks the lacunae seen in the ooplasm of figure 2. × 450. Fixation by B-20.

4 A nearly ripe sexual egg. The egg nucleus has arrived at approximately the stage shown for the parthenogenetic egg in figure 12. In the nurse cells both nucleus and cytoplasm show degeneration, the nuclear degeneration being more advanced than in the egg nucleus. The yolk spherules have arrived at mature condition and maintain this appearance until cleavage is well advanced. The unknown body is in its characteristic position near the edge of one of the nurse cells and opposite the egg nucleus. × 450. Flemming's triple stain.

5 Sexual egg in same stage as figure 4, showing, in addition, the oogonia and oocytes (*Oog.* and *Oo.*). Flemming's triple stain. × 225.

6 Portion of sexual egg (oblique section) at a little later stage of development than in figure 5. The nurse-cell nucleus has disintegrated; the degenerate remains of one nurse cell (*Deg.n.c.*). The division nucleus shows only a small portion of the deeply staining nucleolar substance; the surrounding granular substance of the ooplasm is more deeply stained than the rest. This nucleus has nearly reached the stage corresponding to that shown in figure 18. × 225. Flemming's triple stain.

PLATE 2

EXPLANATION OF FIGURES

Growth stages of parthenogenetic egg. Figures 7 to 11 were drawn at a magnification of 3600; 12 to 20, at 2700; in reproduction they were all reduced one-half. 7 to 9, several ova in each figure; 10 to 20, one ovum each, and showing only a portion of the ooplasm.

7 to 11 Stage 1 in growth series.

7 Four oogonia.

8 Early oocytes.

9 Somewhat older oocytes, showing increase in number and size of nucleolar bodies; achromatin; characteristics of ooplasm.

10 and 11 Much later stages. In figure 11 the nucleolar bodies begin to show degeneration at the periphery. The ooplasm is still unvacuolated.

12 to 17 Stage 2 in growth series.

12 A still later stage than that shown in figure 11, showing a more advanced stage of degeneration of the nucleolar bodies and the appearance of vacuoles in the ooplasm. The nucleus now lies near the periphery, on the side next the alimentary canal.

13 and 14 Exceptional method of nucleolar degeneration which may occur between stages shown in figures 11 and 15.

15 The method of nucleolar degeneration which usually follows condition shown in figure 12.

16 The nucleolar substance very finely divided and almost filling the nucleus. A layer of achromatin is still to be seen next the nuclear membrane.

17 The nucleolar substance has become more finely divided and entirely conceals the achromatin. The contents of the nucleus are apparently homogeneous.

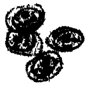
18 to 20 Stage 3 in growth series.

18 Appearance of clear area in nucleus on side toward alimentary canal. In ooplasm, a portion which stains more deeply on opposite side of the nucleus.

19 Appearance of fibrillar structure (spindle?) in clear area and beginning of disintegration of nuclear membrane.

20 Appearance of chromatin on a differentiated spindle; further disintegration of nuclear membrane.

PLATE 2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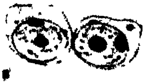
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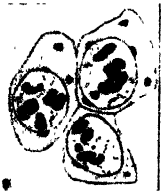
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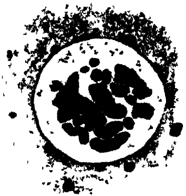
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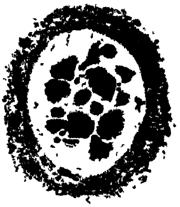
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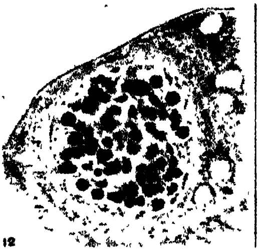
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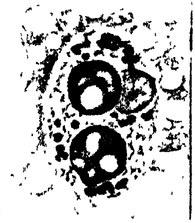
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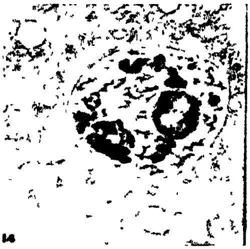
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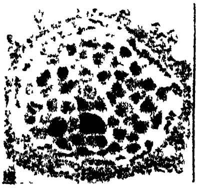
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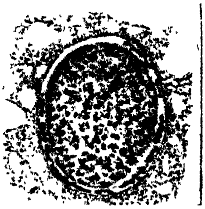
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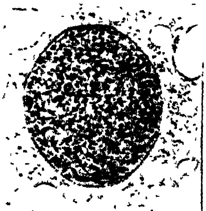
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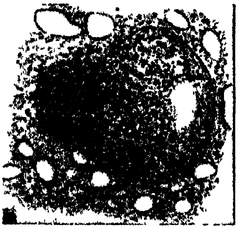
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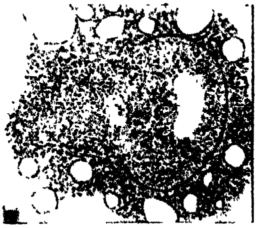
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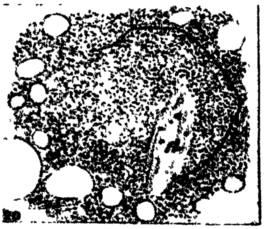
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PLATE 3

EXPLANATION OF FIGURES

The chromosomes in prophase, metaphase, and anaphase, with formation of the polar body. Figure 21, × 600; all others, × 1800. All eggs presumably female-producing.

21 Sexual egg before laying, showing division nucleus (*C.*) near the edge of a bulge in the egg; granular degenerating substance (*G.s.*) (compare fig. 6); spherules of deutoplasm (*D.*).

22 Portion of same section as figure 21 more highly magnified, showing twenty-one chromosomes at metaphase, one about twice the size of the others and interpreted as two fused.

23 to 28 Chromosome complexes in parthenogenetic eggs in metaphase, one lateral and five polar views, all showing twenty-two chromosomes, except figure 25, in which twenty-one are visible, with the possibility of one concealed behind the others or lying in the next section. Figure 26 is drawn from adjacent sections, the chromosomes in each section shown separately. Figures 23 to 25, eggs from mothers killed before laying, immediately after release of young; figures 26 to 28, from eggs just laid. (Temperature, 23°C.)

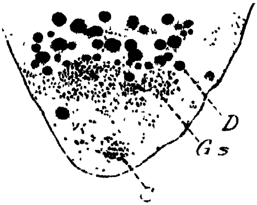
29 Sexual egg just after laying; chromosomes in anaphase, eleven in each set. The chromosomes of one pole differentiated from those at the other by the two methods of drawing.

30 Parthenogenetic egg after laying; late anaphase; from two adjacent sections, the lower two groups in one section at different levels. Note smaller size of chromosomes in polar-body group; only sixteen could be seen; twenty-two chromosomes in the upper two groups. (Temperature, 23°C.)

31 Parthenogenetic egg just laid; from two consecutive sections; polar view. All chromosomes of lower group in one section and at same level; fourteen of upper group at another level, and the other eight in the next section; twenty-two chromosomes at each pole. Six of the polar-body chromosomes are slightly larger than the others, about the same size as shown in the polar-body group in figure 30. (Temperature, 23°C.)

32 Parthenogenetic; three minutes after laying. (Temperature, 17°C.) In this cell not all of the chromosomes could be determined at either pole. Those of the cleavage nucleus were slightly elongated and stained very lightly, having probably passed beyond the deeply staining stage. The polar-body chromosomes have fused into several masses.

33 and 34 Parthenogenetic; two stages in polar-body formation following the stage shown in figure 32; five minutes after laying. (Temperature, 28°C.)



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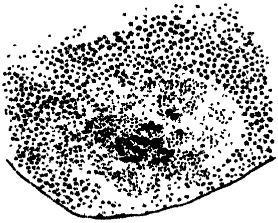

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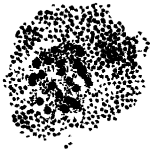

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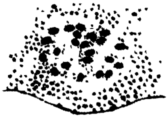

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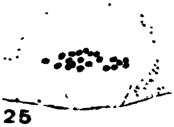

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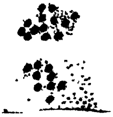

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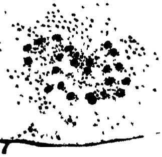

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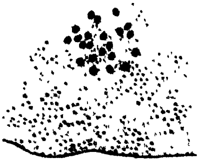

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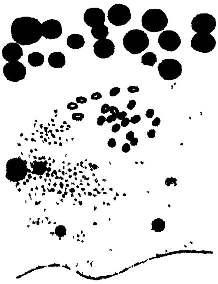

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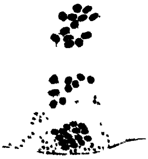

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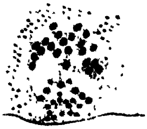

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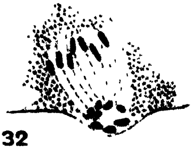

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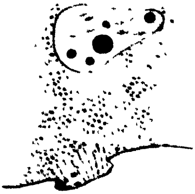

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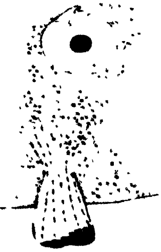

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STUDIES OF PARAJULID DIPLOPODS

I. THE DEVELOPMENT OF THE EXTERNAL SEXUAL STRUCTURES OF PARAJULUS IMPRESSUS SAY(8)

R. A. HEFNER
Miami University

FOUR PLATES (TWENTY-FIVE FIGURES)

AUTHOR'S ABSTRACT

A description of the gross embryology of the sex apparatus of a common diplopod, the corn millipede. The life cycle of this animal involves eleven instars extending over a period of three years.

In the male the true first legs are shed at the ninth ecdysis and the large clasping appendages are substituted through the last three instars In the region of the second body somite the fused coxal plate and the penes are developed at the final molt; the second appendages are reduced at this time. The greatest changes occur in the region of the seventh somite, where the legs are shed about the eighth ecdysis and the gonopods developed through the remaining instars. It is evident that these gonopods arise as a modification of the sternites of the seventh somite.

In the female the second legs are reduced to vestiges at the final molt The structures at the mouth of the oviducts are developed from the surrounding tissues and the modified sternites of the reduced second legs. These changes are in progress as early as the seventh instar, but the greatest changes occur at the final ecdysis.

INTRODUCTION

The family Iulidae, to which the genus Parajulus belongs, is composed of diplopods (Chilognatha, millipedes) of the arthropod class Myriapoda, having a smooth, slender, cylindrical body with a well-developed head and sense organs. The heavy, chitinous, external armor consists, in general, of serial rings composed of ventral sterna fused to laterodorsal scuta. The latter are entirely without projecting lateral lamina.

The genus Parajulus was differentiated from Iulus by Humbert and Saussure(5), in 1869, with the following generic description:

> Related to the Genus Julus. Second joint of the jaws very much swelled and dilated. Lip of female formed as in Julus, with small intermediate, triangular lamella [promentum—Latzel(6)]; in the male the intermediate lamella is large, ovate, with the front interior

layers compressed by it (the intermediate layer). The lobes of the first (outer) segment are lateral; of the male, wide, rounded, or somewhat squared; in the female, narrowed, held together slightly angular. Position of the feet in segments as follows: I—1; II—0; III—0; IV—1; V and the following, 2; in the male segment VII bears the copulatory organs. Feet in the male have no small groove. First pair of feet in the male five jointed, very much swollen, the fourth joint being especially inflated. The fifth joint is armed with a flesh-colored oval swelling.

Bollman(1) would raise the genus to subfamily rank, but such status does not seem to be recognized by later authors. He also adds the following additions and corrections to the above description:

> Mandibular combs 9 or 10 (4 in Iulids); second pair of legs dwarfed; in the male the first pair of legs (six-jointed), the promentum, and the first dorsal scutum very large.

Say's description(6) of P. impressus follows:

> Body cylindrical, emarginate, above brownish, beneath yellowish white, appearing glabrous; segments each with a lateral black spot, whitish lines and dots sometimes obsolete, a transverse series of longitudinal, abbreviated, obsolete, impressed lines; and beneath the stigmata with impressed, more distinct ones, ultimate mucronate, spiracles not prominent; eyes rather large, conspicuous, black; labrum yellowish white; antennae brownish.

This description is not adequate and might apply to P. venustus and P. diversifrons as well as to impressus. This leads to confusion in the literature referring to these species. Wood(11) describes venustus, figures the gonopods of impressus, and mentions diversifrons as a possible variety of a single species. These difficulties were partially adjusted by a later(12) publication. Bollman(1) concludes that venustus has a more northerly distribution than impressus and fixes the type species for Say's name. Nevertheless, Brolemann(3) reverses the specific names for impressus and venustus in a recent paper.

GENERAL EMBRYOLOGY

In all millipedes the sexes are separate and internal insemination of the egg occurs. The external orifices of the genital organs are located anteriorly, and the copulatory organs may sometimes be modified portions of appendages. In addition to the actual genital apparatus, other very marked changes in the anterior region are in accord with reproductory activities, and the constant nature of all these modifications has led to their extensive use as specific diagnostic characters. By the use of these structures the three species above mentioned have been definitely described and figured(10).

The life-stages of P. impressus extend over a period of three years and are marked by a series of ten definite molts or ecdyses of the exoskeleton. Stage I in the table below is the newly hatched hexapod larva and stage XI is the adult; future reference to immature forms will be made as stages here represented. All the features tabulated vary slightly, and most figures are therefore approximate.

STAGE OR INSTAR	NUMBER OF SEGMENTS	PAIRS OF APPENDAGES		LENGTH OF STAGE IN DAYS
		♂	♀	
I	7	3	3	15
II	13	7	7	15
III	17	15	15	15
IV	22	25	25	20
V	29	39	39	30
VI	35	51	51	30
VII	40	63	65	60 or 200[1]
VIII	45	73	75	
IX	48	81	83	60
X	50	89	91	365
XI	51	93	95	285

[1] May overwinter at either stage VII or VIII.

The genital apparatus and secondary sexual characters of P. impressus will be separately described for each sex. The description of each structure will be followed by remarks on its gross embryological development.

MALE

In the adult male the first pair of appendages are enormous, swollen structures, retaining the characteristic six joints in an enlarged and modified form. These appendages project ventrolaterally, then curve anteromedially and extend forward directly beneath the mandibles. It has been presumed that these grotesque structures serve as clasping organs during copulation (figs. 1 and 2). Observation reveals that they are inserted in the grooves between the gnathochilarium and the mandibular stipes of the female, where they are used to pull the head forward, separating the sternites of the anterior somites and fully exposing the vulvae when copulation begins.

The first pair of legs remain unmodified and indistinguishable from those of the immature female in all instars of the male up to, and including, stage VIII. At stage IX the musculature of the first legs is reduced and irregular or entirely wanting, suggesting the action of phagocyte cells. At the same time there is a concentration of material in the region of the sternite, indicating the beginning of the great clasping organs. The illustration (fig. 4) of this stage shows the legs as empty chitinous shells and the indications of the claspers in the form of dense mounds. At the next ecdysis (stage X) the true legs are completely shed, and the claspers appear as short squat projections already showing distinct segments (fig. 3). The change of proportions from this stage to the adult structure at the final molt is accomplished by an increase in size of the segments of the claspers.

The second pair of legs are vestigial in the adult male. The vestiges consist of the four distal segments, none of which is greatly modified. These fragmental appendages are attached to a peculiar chitinous plate which arises in the region where the second sternite should be located, projects ventrad, and then bends almost at a right angle to extend anteriorly in a plane parallel to the main axis of the body. The proximal portion of this plate consists of a pair of broad lateral flanges, which narrow distally and separate medially to form two strong, blunt prongs which extend between the first legs or claspers almost to the gnathochilarium (figs. 1 and 6). The vestigial appendages attach to the sides of the plate just anterior to the right-angle turn and project anteroventrally. Since these rudiments exhibit the four terminal segments of appendages and no separate sternite is visible, it is presumed that the plate is formed by a fusion to the sternite of the proximal two joints of each second appendage. The plate exhibits no markings to indicate the lines of such fusions, however. Just posterior to the perpendicular portion of the plate is a short, bilobed projection which extends ventrad from what should be the sternal region. The lobes are the penes, which retain separate ducts, although fused into a single structure. The function of any portion of this plate, other than the penes, is not apparent. The entire structure arises abruptly at the last molt. Stage X shows normal second appendages attached to an undifferentiated sternite. The shift to the puzzling apparatus described above thus occurs in a single ecdysis.

The third somite is without appendages, the fourth bears one pair, and the fifth and sixth each two pairs, all of which are without sexual features.

On the seventh somite the segmental appendages are replaced by two pairs of highly modified gonopods. Each member of the anterior pair or coleopods is composed of two distinct branches. The anteromedian branch is a thick rounded club-like affair densely beset with projecting bristles. Behind and lateral to this structure lies the second branch, which consists of an irregular chitinous plate rolled inward at the posterior margin to form a partially enclosed gutter or tube (figs. 1 and 7). Both of these branches extend ventrad from the body, and near their junction they are attached to the body wall. Each of the posterior gonopods or phallopods likewise consists of two branches which arise from a common base just posterior to the attachment of the coleopods. The outermost of these branches is a long, slender, flattened tube which projects ventrad from the body in a posterior direction, bends to pass anteriorly between the coleopods, and then curves posterodorsally, making a complete S-shaped figure. Near the common base the second branch arises and spreads into a broad, thin, leaflike structure set vertically at right angles to the main axis of the body. The inner margin of this plate may come into contact with that of the opposite plate. This device probably checks the backward flow of the sperm-bearing fluid, which emerges from the penes on the second somite and proceeds along the ventral surface of the body. The spermatozoa collect at the base of the gonopods, by which they are transferred to the vulvae of the female. It is usually presumed that the phallopods or posterior pair of gonopods act as organs for the transfer of sperm, but the delicate nature of the hollow branch of the phallopod and the distinct grooved structure of the coleopod in this animal suggest that the latter may be the chief agent in the delivery of spermatozoa to the vulvae (figs. 1, 8, and 9). Observations on another species (P. pennsylvanicus) taken in copulation showed many non-motile spermatozoa in the grooves and among the bristles of the coleopods; the phallopods of this species are reduced to vestiges.

The seventh somite usually bears normal appendages at stage VII, but the metamorphosis is somewhat irregular and the ensuing description may sometimes apply to this stage. At stage VIII the appendages of the seventh somite are usually lacking, though a modified rudiment may occasionally be found (fig. 13), and two pairs of segmented, filiform vestiges frequently indicate an abortive attempt to form legs here during metamorphosis (fig. 12). At this stage the sternite of the seventh somite is detached from the scute and exhibits four minute swellings to which the vestigial legs may be attached. The fact that these vestiges may show six segments indicates that these swellings (embryonic gonopods) arise from the double sternite of the seventh segment. Stage IX shows the sternal swellings slightly more pronounced and may still present vestiges of the abortive legs, in the absence of which the point of the former attachment is indicated by protuberances on the modified sternites (fig. 11). In stage X the sternal mounds show some degree of differentiation consistent with the divisions of the adult gonopods, but may still retain the leg scars (fig. 10). The two lobes of the anterior mound are interpreted as the forerunners of club-shaped and tube-like divisions of the coleopod, respectively. It will thus be seen that by far the greatest degree of differentiation in the gonopods (as well as in the previously described structures) occurs at the final molt.

FEMALE

The first pair of legs of the female parajulid are unmodified for copulation, unless being crowded anteriorly by adjoining structures be so considered. The sternite of these appendages is a broad leaflike structure and appears as such in the larval stages of both male and female P. impressus (figs. 5, 18, and 19).

The second pair of legs are delicate, filiform vestiges in the adult female. They are attached to the anterodorsal border of the vulvar mass which constitutes the external female genital apparatus. In the larval stages observed the second legs are but slightly modified, an elongation of the coxae being the only deviation from the normal appendage (figs. 20 to 25, inclusive). Here then, as in the male, the second legs are reduced to vestiges at the final molt.

The vulvae of the adult female parajulids lie between the first and third pairs of legs. These legs belong to the first and fourth somites, respectively, since the third somite is apodous. The vulvae are thus ventral to the second and third somites. In P. impressus they consist of three well-defined parts, the synoperculum and a pair of mounds. The synoperculum is a heavily chitinized double funnel, which extends ventrad and hence perpendicular to the main axis. Oval slits extend transversely on either side of the midline of the synoperculum. The caudal margins of these slits are bounded by triangular plates which are fused medially to form the 'heart-shaped projection.' The cephalic margin is formed by two transverse chitinous plates separated medially by an impressed apical notch and fused laterally to the plates of the heart-shaped projection (figs. 14, 15, 16, and 17). The sternite of the second appendages is without marked distinctions from the usual sternite during larval stages, but at the final metamorphosis it enlarges to become the cephalic border of the adult synoperculum. Small particles of chitin attached to the base of the vestigial second legs are thus not interpreted as being vestiges of the sternite.

The posterior plates which fuse to form the heart-shaped piece at the posterior margin of the opercular funnels in the adult appear as early as stage II as fragments of chitin in the membrane bordering the caudal side of the second sternite. These plates increase in size with each succeeding molt, but do not fuse until the adult stage is reached (figs. 21 to 24, inclusive).

On the caudal side of the vulvae and posterodorsal from the synoperculum are the mounds, chitinized oval structures each composed of two valves partially embedded in the integument ventral to the oviducts (figs. 15 and 16). The function of these structures in copulation or other reproductive activity is at present disputed.

The mounds arise as sheets of chitin in the integument posterior to the tracheal stalks of the second appendages. In early larval stages they appear as irregular paired lobes of unchitinized tissue, situated just dorsal to the posterior plates. This condition is observed as early as stage VII. At each succeeding molt the tissue is progressively chitinized, and in stage X the features of the adult structure are discernible (figs. 21 to 24, inclusive).

DISCUSSION

The subject-matter of this paper is not without controversial features. The usual interpretation of the origin of the external sexual structures of millipedes is that they are modified appendages. The claspers of the male parajulid certainly have the appearance of appendages, but the present observation shows that the true appendages are destroyed and the claspers substituted. This behavior is suggestive of phagocytic action of the type observed in insect metamorphoses.

The penes of the male are characteristically located on the coxite of the second pair of legs in the millipedes. In Parajulus no definite coxite is discernible in the adult male, but the sternite, coxite, and proximal two leg segments are all fused to form the heavy chitinous plate in the region of the second somite. Brolemann(2) solves this problem by calling the portion to which the penes are attached the fused coxites.

The gonopods, as their name implies, are presumed to be feet modified for reproductory purposes. It has been previously shown for a polydesmid millipede (Miley(7)) that such is really the case. In this animal, Euryurus erythropygus, the gonopods have the segmental features of the legs. In Parajulus impressus the gonopods are not only without segments, but are observed to appear before the complete ecdysis of the legs of the seventh somite. Abortive vestiges of the legs may appear when the embryonic gonopods are fairly well differentiated. Since the gonopods are observed to arise in the sternal region and the vestiges of the legs of the seventh somite are definitely attached to the embryonic gonopods, it is imperative that the gonopods be considered modifications of the sternites of the seventh somite.

The entire external female sexual apparatus is located in the region of the second somite. Concerning its origin, Verhoeff(9) and some of his coworkers would derive the vulvae from the region of the third somite, which is without appendages in the parajulids. Opposed to this view is the opinion of Brolemann and Lichtenstein(4), who conclude that the vulvar region is elaborated from the openings of the oviducts on the second somite and the sternal region of the same segment. The observations made in preparation for this paper support the contentions of the latter authors.

ACKNOWLEDGMENTS

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SUMMARY

1. The life-cycle of Parajulus impressus involves eleven instars extending over a period of three years.
2. The claspers on the first somite of the male are developed through the last three instars and replace the legs which occupy the first somite in early larval stages.
3. The penes and the surrounding structures of the second somite appear at the final molt.
4. The male gonopods on the seventh somite are developed through the last four or five instars. They arise from the modified sternites of the seventh somite.
5. Abortive vestiges of the appendages of the seventh somite may appear in one or two instars following the shedding of the true legs. Such vestiges are attached to the embryonic gonopods.
6. The second legs of the female Parajulus impressus are reduced to vestiges at the final molt.
7. The anterior border of the synoperculum arises from the modified sternite of the reduced second legs at the last molt.
8. The heart-shaped plate which forms the posterior border of the adult synoperculum arises in the membrane posterior to the second sternite as early as the seventh instar, but does not fuse with the sternite until the final ecdysis.
9. The mounds appear as chitinized tissue as early as the seventh instar and their development parallels that of the heart-shaped plate.

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ABBREVIATIONS

app., reduced second legs of female
cl., coleopods
clp., claspers
cx., coxite
gnath., gnathochilarium
gon., gonopods
h.s.p., heart-shaped plate
md., mound
m.cd., mandibular cardo
m.st., mandibular stipe
o.d., oviduct
ov., ova
p., penes
pl., chitinous ventral plate of male
pr., promentum
st., sternite
syn., synoperculum
t., tracheal stalks

PLATE 1

EXPLANATION OF FIGURE

All figures are magnified about × 17.5, unless otherwise specified. Figures 1 to 13, inclusive, refer to the male.

1 Ventral view of anterior end of adult male.

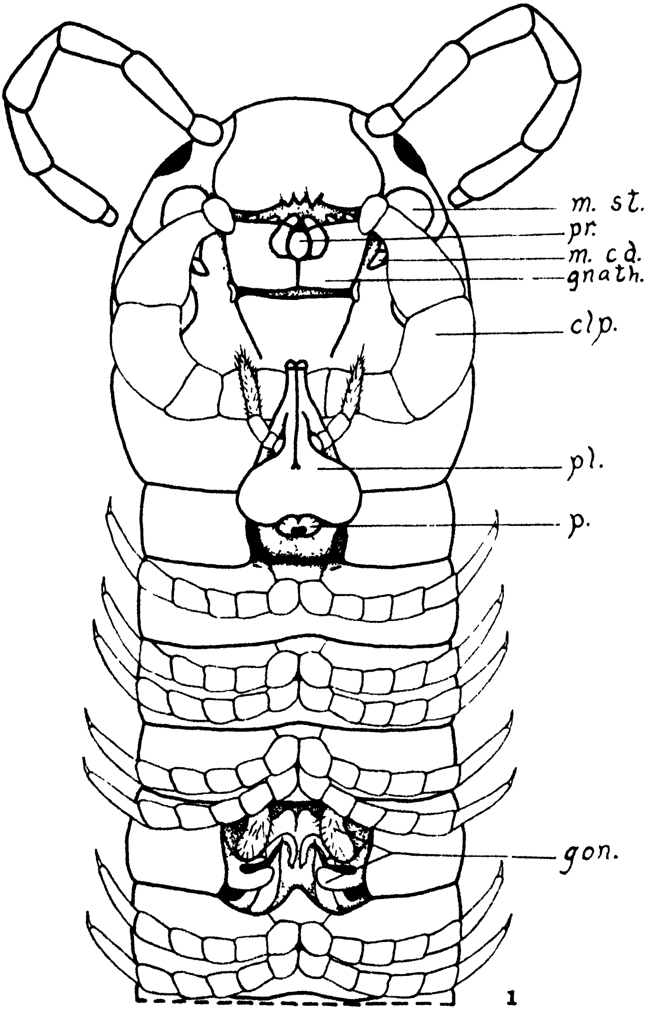

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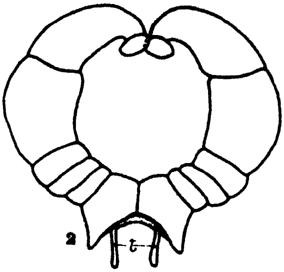
PLATE 2

EXPLANATION OF FIGURES

2 Cephalic view of the adult claspers.
3 Caudal view of immature claspers; stage X.
4 Hollow rudiments of legs and beginning of claspers; stage IX.
5 First legs, showing musculature; stage VIII.
6 Lateral view of ventral plate and rudiment of second leg; adult.
7 Coleopods; anterior view.
8 Phallopods; posterior view.
9 Gonopods; lateral view.
10 Embryonic gonopods; stage *X*.
11 Embryonic gonopods, showing leg scars; stage IX.
12 Embryonic gonopods with vestiges of abortive legs attached; stage VIII. × 35.
13 Irregular metamorphosis of the legs of the seventh somite; stage VIII. × 35.



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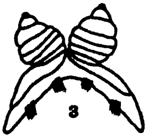

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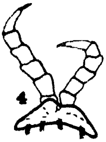

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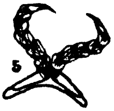

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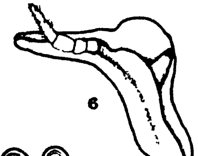

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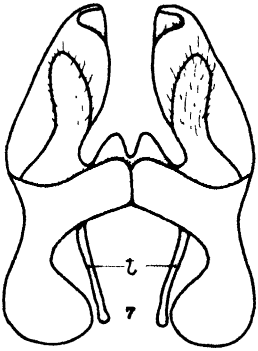

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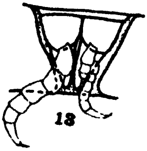

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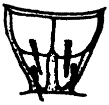

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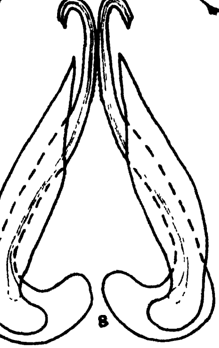

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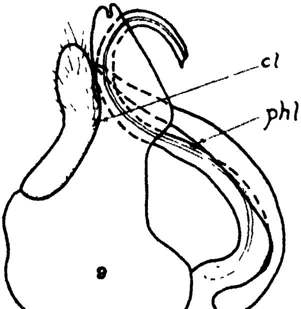

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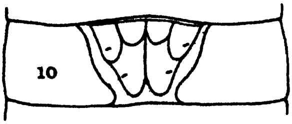

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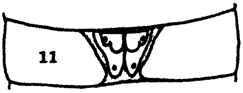

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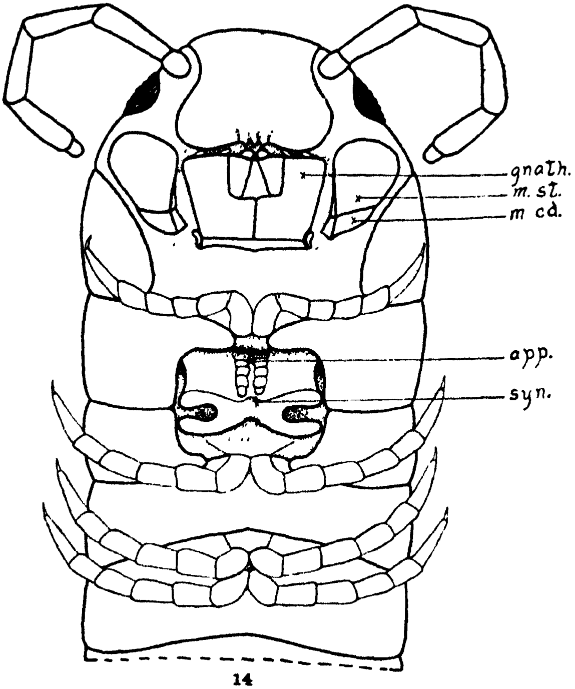
PLATE 3

EXPLANATION OF FIGURES

Figures 14 to 25, inclusive, refer to the female.

14 Ventral view of anterior end of adult female.
15 Lateral view of adult sexual apparatus. × 15.



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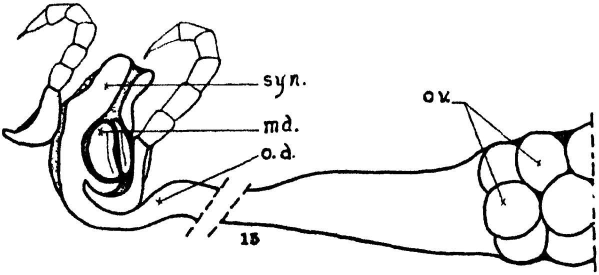

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PLATE 4

EXPLANATION OF FIGURES

16 Caudal view of adult sexual apparatus.

17 Cephalic view of adult sexual apparatus.

18 Cephalic view of pilose first legs of adult. (Hairs omitted in the remaining sketches.)

19 Cephalic view of first legs; stage X.

20 Cephalic view of second legs; stage X.

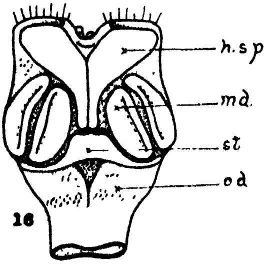
21 Caudal view of second legs; stage X.

22 Caudal view of second legs; stage IX.

23 Caudal view of second legs; stage VIII.

24 Caudal view of second legs; stage VII.

25 Caudal view of second legs; stage VI.



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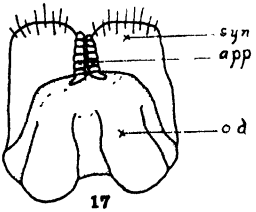

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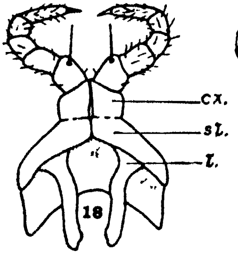

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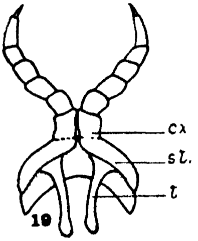

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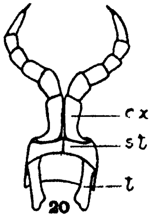

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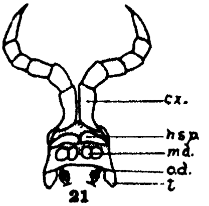

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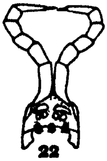

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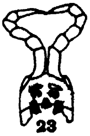

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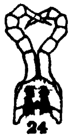

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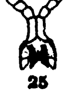

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A CYTOLOGICAL STUDY OF THE PROXIMAL AND DISTAL TUBULES OF THE MESONEPHROS OF NECTURUS MACULOSUS. (UNDER NORMAL AND EXPERIMENTAL CONDITIONS)

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NINE TEXT FIGURES AND NINE HELIOTYPE PLATES (FORTY-EIGHT FIGURES)

AUTHOR'S ABSTRACT

The purpose of this study was to determine whether structural elements of the cytoplasm can be demonstrated to be specifically concerned with certain physiological processes that are experimentally induced in tissue at the time of fixation. The methods for bringing into view variations in cell structure correlated with cell activity were as follows: the increase of the process of secretion by subcutaneous injection into the living animal of phenol red and of urea; the increase of the process of reabsorption by injection of glucose. the inhibition of reabsorption of sugar by injection of phlorhizin.

Variations of the same magnitude in the form, position, quantity, and interrelationships of granules, globules, chondriosomes, and Golgi substance were found to occur in normal untreated animals and in those treated experimentally. Some evidence was found that in cells of the proximal tubule a characteristic form of Golgi body appears which seems to be correlated with increased reabsorption of sugar.

INTRODUCTION

The demonstrated correlation between the chromatin of germ cells and the facts of mendelian heredity encourages continued research for evidence of some indication of causal relation between visible constituents of the cytoplasm of functional somatic cells and specific physiological processes. An investigation of the structural components of the cells of the proximal and distal segments of the uriniferous tubule of Necturus maculosus was undertaken on the assumption that, if the visible structures of these cells constitute the morphological mechanisms involved in physiological processes occurring in the kidney, evidence of this correlation should be revealed if specific physiological processes are experimentally induced and controlled at the time of fixation of the tissue.

The complexity of the organization of the kidney cell is evident. Each cell carries on its own metabolism and, in addition, its special functions of secretion or absorption or both. For each process, it is possible that a distinct structure or organization of structures is differentiated. Two methods for bringing into view the structural expression of specific physiological processes were formulated and carried out: first, the exaggeration of the process of secretion or of reabsorption by subcutaneous injection into the living animal of large quantities of substances believed to be secreted (phenol red, urea) or reabsorbed (glucose); secondly, the inhibition of the process of sugar reabsorption by injection of phlorhizin into the living animal. Under these conditions structural elements of the kidney cell might be expected to deviate from the normal in form, amount, composition, or relative position, if they are specifically concerned with processes by which substances are transported.

The cytological observations were focused primarily upon the form, amount, and intracellular relationships of the following constituents of the cells of the proximal and distal tubules: granules of different kinds, globules, chondriosomes, and Golgi substance.

Reasons for the use of Necturus for this study are: that the proximal segment of the uriniferous tubule of this species has been shown to be the site of reabsorption of sugars and chlorides (White and Schmitt, '26); that a satisfactory fixation of cytoplasmic constituents of cells of the tubule is readily obtained; that the unusually large size of the cells of Necturus facilitates accurate observation of details of structure, and that the cooperation of Doctor White and Doctor Schmitt was assured in suggesting procedures in the experimental treatment of animals supplying tissues for cytological study.

This investigation was suggested by Dr. Caswell Grave, who, in conjunction with Doctor White and Doctor Schmitt, had made a preliminary survey of the problem. The writer

wishes to express sincere appreciation to Doctor Grave for his advice and criticisms, and to Doctor White and Doctor Schmitt for their cooperation.

Acknowledgment is made to Mr. Edward Mallinckrodt, Jr., for his generosity in establishing a fellowship for the purpose of carrying on this investigation in the Department of Zoölogy of Washington University for the year 1926–1927.

REVIEW OF LITERATURE

It is the purpose of these references to published results of physiological and morphological research to show to what extent specific physiological processes have been demonstrated to occur at specific loci in tubules of the kidney, and to what extent these processes may be reproduced by experimental methods and thus made the basis for interpretation of cytological structure of tubule cells. Those papers which are most directly concerned with the material and methods used in this study will be cited.

I. Physiological experiments giving evidence for secretion by tubule cells

A number of physiological experiments have given evidence that excretion of certain substances occurs by means of an active secretory process taking place in the tubule cells. The theory that phenol red is eliminated by secretion through the tubule cells is supported by the work of Marshall and Crane ('21, '22), Marshall and Vickers ('23), Starling and Verney ('25), and Crane ('27). Evidence for the excretion of urea by secretion through the tubules is found by Marshall and Crane ('24), Crane ('27), Starling and Verney ('25), and Holmes and Watchorn ('27).

II. Physiological experiments giving evidence for the elimination of substances according to the filtration-reabsorption theory

Other physiological experiments, however, point to the validity of the filtration-reabsorption theory in explanation of the elimination of these substances. Wearn and Richards ('24), Richards and Barnwell ('27), de Haan ('22) find no necessity for assuming a secretory function of the tubule cells in the elimination of phenol red. Cushny ('26) and Mayrs ('22, '23) find no evidence for the elimination of urea by active secretion, and believe that all results may be explained by filtration and reabsorption.

Especially conclusive are the results of various investigators indicating that sugar is normally reabsorbed from the lumina of the tubules by the cells and apparently returned to the blood stream. Clark ('22), Atkinson, Clark, and Menzies ('21), Starling and Verney ('25) have found evidence for reabsorption of sugar by perfusion experiments. Wearn and Richards ('24), by direct analysis of glomerular fluid as compared with that of urine, prove the reabsorption of sugar and chlorides by the frog kidney. White and Schmitt ('25), using a similar method, demonstrate that the reabsorption of sugar and chlorides by the Necturus kidney occurs in the proximal convoluted tubule. A theory of filtration through the glomerulus, without secretion by the tubules, necessitates the assumption of a process of reabsorption, as is pointed out by Marshall ('26) and Cushny ('26).

III. Morphological investigations that afford evidence for secretion

A number of investigators have made morphological observations that give evidence for secretion. Microscopical observations of cells during the elimination of several dyes have given considerable evidence that the cells secrete dyes into the lumina of the tubules (Heidenhain, '74; Bensley and Steen, '28; Gurwitsch, '02; Höber and Königsberg, '05; Goldman, '09). Evidence that phenol red is secreted is found by Edwards and Marshall ('24) and Bensley and Steen ('28).

Morphological evidence for secretion of urea has been found by Leschke ('15), Chevalier and Chebannier ('15), Oliver ('16, '21), and Walter ('21).

IV. Morphological observations interpreted to suport the filtration-reabsorption theory

Other interpretations, however, have been given to other morphological observations in experiments with dyes, and a number of investigations give evidence supporting the filtration-reabsorption theory. Of these may be mentioned the work of Schlecht ('07), Arnold ('02), Mitamura ('24), Peter ('24), Dawson ('27), de Haan and Bakker ('23). The principal studies giving evidence for the elimination of phenol red by filtration and reabsorption are those of Bieter and Hirschfelder ('24, '26) and von Möllendorff ('15).

Hayman and Richards ('26) question the value of morphological studies to determine the direction of passage of dyes through the tubule cells.

V. Observations concerning the effect of phlorhizin diabetes on the kidney

The effect of phlorhizin diabetes on the kidney is considered by some to indicate an increased secretory activity of the tubule cells (Cullis, '06; Brodie and Cullis, '06, and Brodie, Siau, and Pavy, '03). The results of more recent investigations, however, indicate a decreased reabsorptive function of the cells (Cushny, '26; Mayrs, '23). The view that phlorhizin renders the cell incapable of reabsorbing sugar is well substantiated by the fact that sugar has been demonstrated to be normally reabsorbed (Wearn and Richards, '25; White and Schmitt, '26).

VI. Cytological studies

Many of the earlier descriptions of cytological observations are not mentioned in this review, because of the absence in them of correlation of structure with physiological states, and because in many cases they are based upon poorly fixed tissue. A few papers, however, report results comparable with observations made in this study.

A. Golgi material. Jasswoin ('25) and Avel ('24) find variations in the position of the Golgi substance which they interpret to be an indication of the direction in which materials are passing through the cells. Nassonov ('24, '26, '27), however, believes that the observed variations in position of Golgi bodies may be ascribed to differences that normally occur in different individual animals. In the epididymis of various vertebrates he describes two kinds of secretion as having a common origin in the Golgi material, one migrating to the base, the other to the distal end of the cell. The illustrations of his papers show configurations somewhat similar to some of those seen in the kidney cells of Necturus.

Dawson ('27) states that he was unable to impregnate Golgi bodies in the distal-tubule cells of Necturus, and that the Golgi bodies of the proximal-tubule cells have a more or less basal position. He asserts that too little is known of the Golgi 'apparatus' to afford a basis for judgment of whether it is indicative of reabsorption or secretion in the renal cells.

Turchini ('27) contends that the polarity of a cell cannot be determined by the position of the Golgi 'apparatus.'

Basile ('15) describes the dispersion of the Golgi substance during increased cell activity following unilateral nephrectomy.

A few of the more recent theories proposed regarding the function of the Golgi material are of interest in view of the results of the present investigation. Bowen ('23–'26) shows that, in gland cells of many kinds, the topographical relationship of the Golgi 'apparatus' to secretion granules is such as to suggest a possibility of causal relation between them. He refers to the 'apparatus' as a synthetic intermediary in the process of secretion.

Nassonov ('23–'27) is convinced that Golgi material is a constant, living, active cell structure and that it functions as a secretory organ of the cytoplasm. Secretion granules form within the 'apparatus,' then become free, and ultimately fill the whole cell. He conceives the Golgi 'apparatus' to be a selective agent for the concentration of substances from which secretion granules are formed.

Walker and Allen ('27), experimenting with artificial cells treated by osmic-acid methods of fixation, report results that, if verified, apparently nullify the interpretation that Golgi material is an active cell organ. In their preparations they find structures apparently identical with the Golgi material found in animal cells. They believe their results throw doubt upon the status of the Golgi material as a true organ or structure of the living cell.

B. Chondriosomes. Studies of the chondriosomes of the kidney have been comparatively few, especially in recent years, and, since they have for the most part been purely descriptive in nature, make no contribution toward the interpretation either of renal function or of the nature of chondriosomes. A few investigators have used kidney cells for a study of the function of chondriosomes and their relation to ergastoplasm and plasma fibrils. Studies of this kind have been made by Trambusti ('98), Benda ('03), Policard ('05), Champy ('09).

Numerous papers have been published concerning chondriosomes, their relation to secretion products of various kinds of cell, and changes occurring in them under certain conditions. Only the small number which describe conditions resembling those found in the kidney cells of Necturus are mentioned below.

Regaud ('08, '09) believes the inverse ratio which he finds between the number of globules and mitochondria in the proximal-tubule cells of the kidney is indicative of stages of cell activity. He suggests the origin of globules from mitochondria.

Ma ('20), from a study of stomach cells of various mammals, concludes that no one cytoplasmic constituent can be considered exclusively the parent of zymogen granules. He finds that zymogen granules vary reciprocally in amount and are closely associated in their origin with both mitochondria and Golgi bodies. He interprets this to indicate that both mitochondria and the Golgi 'apparatus' are concerned in the production of zymogen granules.

Policard ('10) made an extensive investigation of the constituents of kidney cells of the frog and their relation to excretion. After feeding frogs with beef, he found no parallel relation between the quantity of mitochondria and changes in the number of granules, and concludes that, although chondriosomes may play an indirect rôle in the formation of granules, no constant genetic relationship exists. Upon removing the liver, however, various modifications occur in the kidney cells which he considers indicative of extreme activity. The chondriosomes break into fragments; globules enlarge by fusion, and at the same time become stainable by plasma stains. He finds variations in chondriosomes of distal-tubule cells during diuresis, and concludes that the distal tubules have to do with the elimination of water. He believes that phlorhizin acts specifically on the chondriosomes of the proximal-convoluted-tubule cells. From his studies he further deduces that the chondriosomes perform a constant function in the cell, and that granulation of chondriosomes is probably due to pathological conditions.

Barratt ('13) studied chondriosomes and granules of the proximal-tubule cells of the kidney during severe hemoglobinemia, and found a great increase in the number of globules, which he interprets to be indicative of extreme activity.

Of importance in the interpretation of results obtained in this study are the observations of Cowdry ('18) that changes in the form of mitochondria constitute the most delicate indications of cell injury. He considers that polymorphism of mitochondria found in cells of the same type is usually the result of experimental procedure before fixation, and he calls attention to variations occurring as a result of unequal penetration of constituents of fixatives, differences in the degree of mordanting and differentiation. However, he cites instances of variations in amount of mitochondria with altered activity and pathological conditions, which he considers significant.

C. Globules. Thomas and Dragoiu ('27) describe an unusual permeability of kidney cells of the young guinea-pig after ingestion of pentose. They find an increased activity of the cells, as shown by the formation of large granules at the bases of the cells. Later, the cells become enlarged and filled with large globules, and finally reduced in size and empty.

D. Brush border. Several investigators have made observations which indicate that the brush border changes in appearance during stages of cell activity (Van der Stricht, '91; Théohari, '99; Tribondeau, '02, and Policard, '10). Peter ('24), however, considers that variations in the appearance of the brush border are probably effects of handling the tissue.

SUMMARY OF LITERATURE

The results and conclusions of investigators, whose works have been consulted, are so conflicting that conclusive evidence concerning the direction or mechanism of passage of substances through the tubule cells is not afforded. The relation of cell structures to these physiological processes is even less well established.

Inconsistencies in physiological as well as in morphological results may in part be due to differences in the several species of animals utilized for study. The only investigation strictly comparable in material and methods with this study is that of White and Schmitt ('26), who demonstrated that the site of reabsorption of sugar and chlorides in the Necturus kidney is the proximal convoluted tubules.

Conclusions which seem to have the greatest validity and which have formed the basis for this investigation are enumerated as follows:

1. Urea is probably secreted by the cells of the proximal convoluted tubule.
2. Phenol red is eliminated by the active secretion of the proximal-convoluted-tubule cells.
3. Glucose is normally reabsorbed by the proximal-convoluted-tubule cells, especially by the part nearest the glomerulus.

4. Phlorhizin prevents the reabsorption of glucose.
5. Golgi substance may indicate by its position the polarity of cells and the direction of passage of materials.
6. The form of Golgi substance may differ in accordance with activity of the cells.
7. Chondriosomes may have some relation to definite physiological processes.

MATERIAL AND METHODS

I. Source of material

The Necturus that supplied the kidney tissue used for study were obtained at various times between October and May from the Chicago Biological Supply House and from a dealer in Oshkosh, Wisconsin. Some animals became infected in the body cavity following injury in the oesophagus due to the presence of a large hook with which they had been caught. None of the animals was kept under laboratory conditions more than a few days.

II. Preparation of animals

The necessity was recognized for the establishment of normal structural configurations of proximal- and distal-tubule cells. These would then serve as a basis for the recognition of variations in structure that may occur in these cells as a result of the increase or inhibition of specific physiological processes induced by experimental treatment of living animals immediately preceding the fixation of tissue. Provision was made for the construction of such a base line of kidney-cell structure by tissue taken from thirty untreated normal animals, from which blocks of kidney were removed and fixed immediately after pithing.

The substances, by the use of which increase or inhibition of specific physiological processes was induced in the cells of the kidney at the time the tissues were taken, are as follows:

A. Phlorhizin was injected, with the purpose of inhibiting the process of reabsorption of sugar by proximal-tubule cells.

In the same experiments glucose was injected, in order that elimination of sugar by the glomerulus might be assured (White and Schmitt, '26), thus presenting the conditions for reabsorption of sugar by the tubule cells; and also in order that the production of glycosuria might be more certain.

B. Phlorhizin was injected without glucose, for the purpose of determining especially whether glycosuria would occur without adding to the normal amount of sugar contained in the plasma. If glycosuria should occur, the tissue would be expected to show the same structural characteristics as those of animals treated with both phlorhizin and sugar.

C. Glucose without phlorhizin was injected, for two purposes: first, that of comparing the appearance of cells perhaps having structural configurations correlated with large amounts of sugar that is reabsorbed, with the appearance of cells from which glucose is excluded by the action of phlorhizin; secondly, that of determining whether glycosuria could be produced by injecting large amounts of glucose.

D. Two substances were used with the purpose of increasing secretory activity of the cell:

1. Urea, to increase the plasma content of a substance normally transported through kidney cells.

2. Phenol red, to introduce into the animal a substance for excretion with which the kidney cells normally have no contact. The reasons for the selection of phenol red are: that it is easily diffusible and is non-toxic, that it is the dye most commonly used in physiological investigations, and that in these investigations phenol red has been shown to be eliminated by an active secretory function of the proximal-tubule cells.

III. Fixation and stains

Blocks of tissue cut in the transverse plane of the kidney were taken from the pelvic portion of the kidney of each animal and fixed with Regaud's formalin-bichromate mixture, Champy's fluid, and by the Mann-Kopsch osmic-acid method. The Kolatchev method was found unsatisfactory for impregnation of the Golgi substance.

The tissue fixed in Regaud's and Champy's fluids was stained by the Bensley-Cowdry acid-fuchsin-methyl-green method. This is considered more satisfactory than iron-haematoxylin in that it stains chondriosomes and large globules red, small granules and cytoplasm green. Sections of material fixed by the Mann-Kopsch method, if blackened too intensely, were differentiated under the microscope with 0.25 per cent $KMnO_4$. The time necessary for differentiation varied from two to twenty seconds.

After pithing the animal, pieces of kidney were removed and put into the killing fluid as quickly as possible, in order to avoid postmortem changes in the cells due to lack of blood supply and exposure to air. The interval of time between pithing the animal and placing the tissue into the killing fluid was not more than three or four minutes, that of actual exposure of the kidney not longer than about twenty to thirty seconds. Five attempts were made to fix tissue without exposure to air, by injecting Regaud's fluid into the body cavity of the living animal, but the results were relatively unsatisfactory, probably due to dilution of the fixative by the body fluid.

IV. Experimental procedures

A. Injections with phlorhizin and glucose. It was found most satisfactory to make a suspension of phlorhizin in olive oil for injection. The exact amount of phlorhizin injected in each case cannot be stated, because, after the syringe needle is withdrawn, some of the fluid leaks from the opening. In animal no. 25, 0.4 gram of phlorhizin in 2 cc. of olive oil per kilogram of body weight was injected subcutaneously in the pelvic region of the animal. For all other animals, about 0.15 to 0.2 gram of phlorhizin per kilogram of body weight was used.

Twelve to eighteen hours after the first injection, the animals were injected subcutaneously with 2 cc. of 4 per cent glucose in distilled water. This also leaked slightly following the withdrawal of the syringe. The animal was then permitted to swim about normally for from twenty-five to fifty-five minutes (in the case of animal no. 86 the time was 105 minutes), and then pithed and nailed to a board. A capillary glass cannula was inserted into each ureter, if possible (this was usually more difficult in females). An interval of about one-half hour usually elapsed before enough urine had collected to test for the presence of reducing sugar. The gills were kept wet with tap-water. The tissues were kept moist with Ringer's solution without glucose. The fluid in the capillary cannula was blown upon and mixed with a drop of Benedict's solution on a clean glass plate, and the mixture drawn into the capillary tube. After the end was sealed, the capillary tube was heated in boiling water to test for the presence of reducing sugar. If urine was present in the bladder, this was tested instead of inserting cannulae. This procedure was carried out in a sufficient number of cases to establish the fact that glycosuria occurred under these conditions. In order to avoid delay before removing tissue, the test was then omitted. Phlorhizin alone causes a glycosuria, as shown by the tests made upon the urine of animals no. 47 and no. 49. A positive test was never obtained with the urine of untreated animals. Observations were made on tissue of eleven animals treated according to this procedure.

Animals nos. 12, 13, 19, and 45 were injected with phlorhizin in Na_2CO_3 and with glucose in the solution. Negative Benedict's tests were obtained in animals no. 12 and 45, and no urine was obtained from nos. 13 and 19.

B. Injections with phlorhizin. Five animals were treated in the same way as those just described, except that injection of glucose was omitted. A positive test for reducing sugar in the urine was obtained in two of these, no. 47 and no. 49. Blocks of kidney were taken from the others without delaying for the test.

C. Injections with glucose. Material was taken from nine animals which had been injected with glucose only (2 cc. of 4 per cent glucose). The urine from only one of these gave a positive test for reducing sugar. This (animal no. 140) was injected three times during a period of one and one-half hours. Animals no. 138 and 139 were treated similarly, but no urine was obtained. Animals no. 134 and 135 were injected twice, but no. 135 gave a negative test with Benedict's solution. In animals no. 8, 9, 51, 132, and 133 one injection only was given, and no urine was obtained. Probably no glycosuria occurred, but, theoretically, the reabsorptive action of the cells should have been increased.

D. Injections with urea. Fourteen animals were injected subcutaneously with 2 cc. of 10 per cent urea solution. Intravenous injection was tried in other animals, but the method was abandoned because of the extra time required for manipulation after pithing the animal and exposing the kidney. The tissue was removed and fixed forty to fifty minutes after the injection.

E. Injection with phenol red. The phenolsulphonephthalein used for injection is the monosodium salt solution prepared in sterile ampules by Hynson, Westcott & Dunning. It was diluted with Ringer's solution so that the amount of dye injected into each animal was about 1 mg. for each 100 grams of body weight. The solution leaked to some extent after the withdrawal of the needle. Tissue was removed twenty-five to sixty minutes after the injection. In a few cases cannulae were inserted into the ureters, and a very small amount of urine obtained. This was put into a drop of NaOH to test colorimetrically for the presence of phenol red. In other cases a test was made on bladder urine, for which a positive test was invariably obtained. The tissue of fourteen animals injected with phenol red was studied.

F. Material from a few infected animals was studied. Three of these belonged to the untreated animals: no. 42, slightly infected, and no. 38 and no. 52, much infected. Three others were treated with phenol red, phlorhizin and glucose, and urea, respectively. It is not expected that significant conclusions regarding the effect of infection of the body cavity upon the kidney may be drawn from these few examples.

G. To test the effect upon cytological structure of lack of blood supply, exposure to air, and injury due to cutting, the following procedure was carried out: Blocks of kidney were taken from normal animals and quickly dropped into the fixative. The animal was then left with the remaining portion of the kidney exposed, and, after an interval of fifteen, thirty, or sixty minutes, other blocks were removed and fixed.

V. Method of cutting sections

The uriniferous tubules of Necturus extend in parallel series between the lateral and medial borders of the kidney. To obtain transverse sections of tubules, the blocks of kidney were sectioned in the plane of the longitudinal axis of the animal. Sections were mounted in continuous series, in order that the position in the kidney of the various diversities in cytological structure might be ascertained.

VI. Evaluating and recording facts

Because the personal factor in cytological studies is so apt to enter significantly into observations of specific structural details and especially into interpretation of cell organization, a special effort has been made to reduce this source of error. Record was made of the size, sex, activity, and experimental treatment of each animal, and of the technique with which the tissue was taken and subsequently treated; but reference to this record was avoided during the period of microscopical observation of tissue. When all records of observations had been completed, the noted characteristics of the tissue were arranged in tables according to the several factors in which structural details were found to vary. The results thus tabulated were then compared with the records of conditions of animals and the treatment of tissue.

The possibility of error in interpretation due to a too restricted number of observations has also been recognized, and, as far as possible, a number of animals considered ample to yield significant results has been used. Many instances of cytological papers have been found in which no mention is made of the number of animals upon which observations have been made. Conclusions which may be clearly indicated by observations of structures found in material derived from a single individual frequently are shown to be without validity when compared with the same structures in corresponding material from other individuals of the same species. Pertinent facts concerning the physiological state of the animals and of the tissue upon which observations have been made are also regarded as important factors to be considered in evaluating the results.

In every investigation of this kind, cases of diversity in structures occur that may be interpreted as artifacts, and when they do not conform with the theoretical system that has gradually crystallized in the mind of the observer, the tendency is to discard them without mention; yet they may be the facts that show the inadequacy of the theory. If tissues prepared in the same way vary widely in certain appearances, the differences may be due to unknown factors in the physicochemical states of the tissue, or to unknown processes involved in fixation. In this study the attempt has been made to subject all observations to critical analysis.

The following table indicates the total number of animals used in each particular procedure and the special number by which each individual was designated; and it records conditions of treatment of animals and of the removal of tissue considered likely to affect cell structure or organization, and the results of urine tests

TOTAL NUMBER OF ANIMALS	PHYSIOLOGICAL STATE	NUMBERS DESIGNATING INDIVIDUAL ANIMALS	FACTORS OF EXPERIMENTAL TECHNIQUE POSSIBLY INFLUENCING TIME OF EXPOSURE OF KIDNEY	RESULTS OF TESTS OF URINE
Untreated animals				
30	Normal activity		Tissue fixed immediately after pithing and opening animals	
1	Normal activity	40	Slightly delayed fixation; urine obtained from bladder	No reducing sugar
1	Normal activity	35	Urine obtained by cannulae inserted into ureters	No reducing sugar
1	Normal activity	60	Regaud's fluid poured upon kidney before removal of tissue	
4	Normal activity	129, 130, 136, 137	Regaud's fluid injected into body cavity of living animal	
7	Normal activity	1a, 2a, 141a, 144a, 145a, 146a, 151a	Blocks of kidney removed after long delay	
1	Slightly infected	42	Tissue taken immediately after pithing and opening animal	
2	Badly infected	52, 38	Tissue taken immediately	
Animals treated with phlorhizin and glucose				
2	Normal activity	25, 34	Urine obtained by cannulae inserted into ureters	Reducing sugar
1	Normal activity (phlorhizin, in Na_2CO_3)	12	Urine obtained from bladder	No reducing sugar
2	Normal activity (phlorhizin, in Na_2CO_3)	13, 19	Tissue taken immediately; no urine obtained	
2	Normal activity	32, 33	Cannulae inserted into ureters; no urine obtained	
6	Normal activity	44, 86, 103, 105, 115, 116	Tissue fixed immediately after pithing and opening animals	
1	Slightly infected, but active	85	Tissue fixed immediately	

TOTAL NUMBER OF ANIMALS	PHYSIOLOGICAL STATE	NUMBERS DESIGNATING INDIVIDUAL ANIMALS	FACTORS OF EXPERIMENTAL TECHNIQUE POSSIBLY INFLUENCING TIME OF EXPOSURE OF KIDNEY	RESULTS OF TESTS OF URINE
Animals treated with phlorhizin				
1	Normal activity	47	Urine obtained by cannulae inserted into ureters	Reducing sugar
1	Normal activity	49	Urine obtained from bladder	Reducing sugar
2	Normal activity	82, 83	Tissue removed immediately after pithing and opening animals	
Animals treated with glucose				
4	Normal activity	8, 9, 132, 133	One injection. Tissue fixed immediately after pithing and opening animal	
1	Normal activity	51	One injection. Urine obtained from bladder	No reducing sugar
1	Normal activity	134	Two injections. Tissue fixed immediately	
1	Normal activity	135	Two injections. Urine obtained from bladder. (Tissue not sectioned)	No reducing sugar
2	Normal activity	138, 139	Three injections. Tissue fixed immediately	
1	Normal activity	140	Three injections. Urine obtained from bladder	Reducing sugar
Animals treated with urea				
13	Normal activity		Tissue fixed immediately after pithing and opening animals	
1	Slightly infected	108	Tissue fixed immediately	
Animals treated with phenol red				
7	Normal activity	67, 68, 70, 71, 111, 113, 127	Urine obtained from bladder	Positive test for phenol red
2	Normal activity	53, 64	Urine obtained by cannulae inserted into ureters	Positive test for phenol red
1	Normal activity	124	Urine obtained from bladder	Negative test for phenol red
3	Normal activity	122, 123, 125	Tissue fixed immediately after pithing and opening animals	
1	Slightly infected	74	Tissue fixed immediately	

OBSERVATIONS

Cells of the proximal portion of the uriniferous tubule of Necturus contain many structural elements that occur in varying degrees of abundance and in varying relationships to each other. Variations in some constituents seem to be related to the position of the cell in the kidney. Facts concerning the amount and structure of these elements, their position in cells, their distribution in the kidney, changes in their structural appearance in adaptation to physiological function, and modifications in their structure due to fixation effects are brought together in the following account.

The diversities of structural configuration in proximal-tubule cells shown in tissue from a single animal, in a single section, and sometimes in a single tubule suggest as a working hypothesis that these cells are the seat of a cyclic process of activity. It is possible to arrange cells which perhaps represent stages in such a cyclic process in a series showing gradations in the number and amount of globules, granules, chondriosomes, and Golgi substance. Such a series of cells is described in connection with descriptions of chondriosomes and Golgi substance.

Cells of the distal portion of the tubule are much less complex in apparent structure than those of the proximal portion, and, as the descriptions that follow indicate, diversities do not occur in sufficient magnitude to suggest stages in a cyclic process.

The presentation of detailed observations will be made in the sequence of the following outline:

I. Regional differences of the kidney.
II. Proximal-tubule cells.
    A. Shape and size.
    B. Abnormal proximal-tubule cells.
    C. Structural constituents.
        1. Brush border.
        2. Globules.
        3. Granules.
            a. Small spherical granules.
            b. Minute black granules.

        4. Crystals.
        5. Vacuoles.
        6. Hyalin body.
        7. Chondriosomes and their relation to granules and globules.
        8. Golgi substance and its relation to granules and globules.
III. Distal-tubule cells.
    A. Shape and size.
    B. Structural constituents.
        1. Chondriosomes.
        2. Golgi substance.
IV. Tissue of infected animals.

I. Regional differences in sections of kidney possibly due to fixation effects

Judgment concerning fixation effects has been based upon the general appearance of the tissue and of the individual cells. If the cytoplasm has a mottled, coagulated appearance, or if the cells appear crushed or shrunken and irregular in shape, the tissue is considered poorly fixed. These appearances of poor fixation are usually visible with the low-power objective of the microscope.

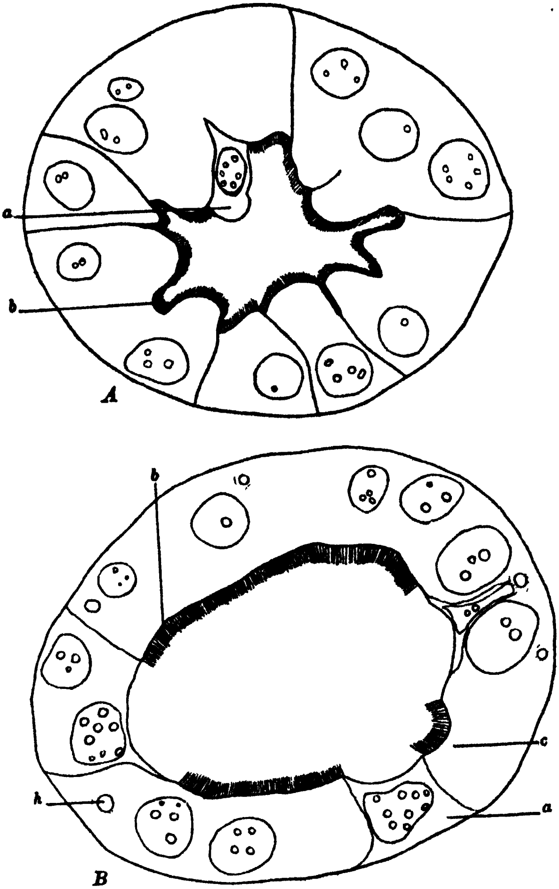
Sections of material killed by all the methods employed, but especially Champy's fluid, exhibit concentric zones of fixation effects, ranging from good at the periphery to poor at the center of the sections. Similar variations in appearance are also found in serial sections, those from the relatively thin lateral edge of the kidney showing better fixation than those of the median portion, where the kidney is thicker and in close contact with the kidney of the other side. Poor penetration of the fixative might be expected to be eliminated by the use of smaller blocks of tissue, thus facilitating more rapid penetration, but since the cells at cut edges are injured, the entire block, if too small, is thus affected. In some entire blocks, however, regardless of the method used, the fixation is found to be excellent, in others manifestly poor throughout.

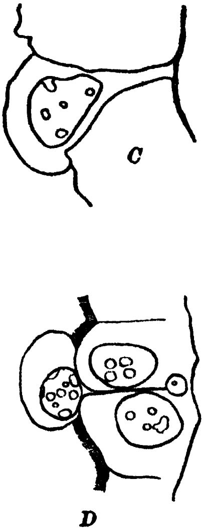
II. Proximal-tubule cells

A. Shape and size. The cells of the proximal tubule differ in shape according to their height. When high they bulge into the lumen, which consequently is small. Sometimes a deep recess separates the swollen inner ends of adjacent cells (text fig. A). The tubules with large lumina are constituted of low cells which tend to have a somewhat concave inner border (text fig. B). Distinct correlation between the size of cells and their structural constituents was not found. The larger cells, however, are usually those that contain many large globules (fig. 10). The smaller cells are more frequently those without globules (fig. 1), but some were found that contain them (fig. 11).

B. Abnormal proximal-tubule cells. In tubules in which the cells are low or of intermediate size a deep concavity in the distal edge of a cell sometimes occurs (text fig. B; pl. 9). Cells exhibiting this character usually have a very distinctly striated brush border. This condition might be attributed to shrinkage of the cell due to fixation, but the tubules containing them show no other signs of shrinkage. It is observed that a cell with this peculiarity is usually found adjacent to a cell of unusual appearance in that it contains no large globules, usually little or no Golgi material or chondriosomes, has no brush border, and takes a lighter stain than surrounding cells (text fig. B; pl. 9). Cells of this latter kind are frequently seen to extend over and obliterate the brush border of adjacent cells (fig. 48). The concavity in the brush border of the normal cell is probably the result of pressure by the adjacent abnormal cell. In portions of the tubule in which the epithelium is higher, the abnormal cells may be long and narrow or triangular in shape, squeezed between two large normal cells, or they may protrude and finally be forced into the lumen (text figs. A, C, D). A process thus appears to be indicated whereby cells, possibly necrotic, are extruded into the lumen.

No regularity in the frequency with which these two kinds of cell occur is evident. Those with concave inner edge are relatively infrequently observed; but the abnormal cells that extend over the inner edges of other cells or are extruded are present in tissue from all animals, both treated and untreated. A greater number of both of these types of cell is found in the tissue of animals no. 134 and no. 138 (fig. 48) and perhaps





Text figs. A and B Outlines of proximal tubules drawn with camera lucida, showing variations in size of tubules and cells. × 680. *a*, abnormal proximal-tubule cell; *b*, brush border; *c*, cell with concave border; *h*, hyalin body.

Text figs. C and D Portions of tubules showing stages in extrusion of abnormal proximal-tubule cells. × 680.

of animal no. 139 than in any others. Since these animals are of those injected with large amounts of glucose, it might be interpreted that the presence of these cell types has some relation to sugar reabsorption. Since other animals similarly treated do not show a great abundance of these two cell types, however, evidence for this correlation is insufficient.

C. Structural constituents. 1. Brush border. Many aspects of the brush border are found in all the material studied. Several observations lead to the conclusion that it is probably present and evenly striated in all stages of normal activity of the cell.

a. A perfectly formed and characteristic brush border may be found in all the types of proximal-tubule cell that have been distinguished.

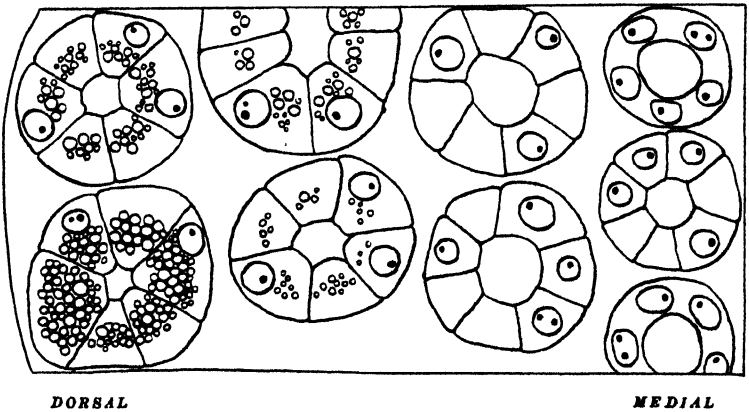
b. The structural appearance of the brush border is affected by fixation. Its characteristic form of regular parallel striations appears most frequently after fixation by the methods of Mann-Kopsch (pls. 4, 5, 7, 9) and Champy. After fixation with Regaud's fixative, it more often lacks striations and evenness of form, and appears coagulated (pls. 1 and 2).

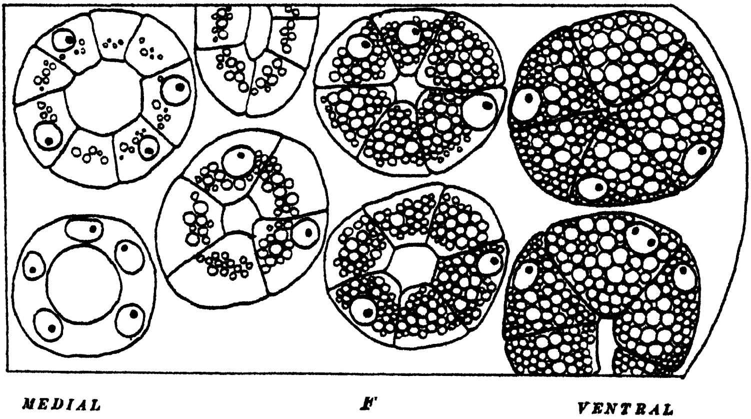
c. When the striations of the border are not evident, in its place is often found an irregular homogeneous distal edge that is interpreted as the coagulated remains of a brush border (figs. 10 and 37). This condition is found in all types of proximal-tubule cell, but is more often found in the type of cell characterized by unusual height and by an abundance of large globules. This is the only indication that the brush border tends to be modified during certain stages of cell activity.

d. The brush border of cells of treated animals shows the same variations as those of untreated animals.

e. Whether evenly striated or coagulated and distorted in shape, the brush border cannot be used as a sure criterion of good or poor fixation, for a cell with coagulated brush border may otherwise have excellently fixed cytoplasm, and, on the other hand, a cell with a perfect brush border may show effects of shrinkage in its cytoplasm. Moreover, no evidence is found that coagulation of the brush border is due to postmortem changes, since it is often found even and distinct in tissue the fixation of which has been long delayed after exposure of the kidney.

2. Globules. Large cells characterized by their content of globules occur in the proximal segment of the tubule (figs. 10 and 37). The number and position of these cells are constant for each individual animal, irrespective of the method of





F

Text figs. E and F Diagrams of a portion of a section of kidney, showing the relative distribution of gradations in the number of globules contained in the cells of the proximal convoluted tubules. × 680.

fixation. In material prepared by Regaud's and Champy's methods and stained by acid fuchsin and methyl green, the globules of largest size take a brilliant red stain, those of smallest size a dark purplish (as if red and green were mixed) or even a greenish stain; while those of intermediate sizes are stained in gradations of shades of red intermediate between these two extremes, increasing clearness of the red being roughly correlated with increasing size of the globules (figs. 9 and 10). In material fixed by the Mann-Kopsch method globules assume various shades of light brown, depending on the degree of differentiation by the potassium permanganate (figs. 24, 25, 26, 27, and 37). Large globule-filled cells are nearly always more swollen and contain globules that are larger in size, or greater in number, when located at or near the periphery, than cells located nearer the center of the kidney. They are usually found in relatively greatest number on the ventral side of the kidney (text figs. E, F), and in some blocks of tissue they are present on the ventral surface only. The first several sections of a series, beginning at the lateral edge of the kidney, also often contain cells of this type only. Sections from the interior of the kidney become larger in area, and contain progressively fewer of the globule-filled cells.

The experimental treatment to which the tissue was subjected seems to have no causal relation to the presence of these globules in greater or smaller number. In all except two of the animals subjected to treatment in which a slight or great delay occurred between the opening of the animal and fixation of the kidney, globule-filled cells are present in great number; and in these two exceptions fixation was but slightly delayed. In the greater number of cases in which tissue was taken without delay, however, as many, and sometimes relatively more, large globule-filled cells are present than in cases of delayed fixation. No correlation between the number of globule-filled cells and the size or sex of the animal or the season of the year is found. These statements are true both for control animals and for those treated with various substances (table 1).

A number of interpretations may be suggested in explanation of these observations. The condition may be a normal one, due to differences in activity in cells of regions of the tubule located at the surface as compared with regions situated in the interior of the kidney. For example, primary tubules, which are the first to develop, open into the body cavity by a nephrostome, and which form a more or less superficial layer at or near the ventral surface of the kidney, may have a specialized function. Chase ('23) states that the region of the convoluted tubules, following Bowman's capsule and the ciliary neck, courses toward the periphery of the kidney. In the sections used for study, however, it has not been possible to trace individual tubules for the purpose of determining definitely this relationship and its correlation with the presence of cells containing large globules. Such a fact, if established, would be significant in view of the work of White and Schmitt ('26), who, by direct analysis of fluid taken from the glomerulus and proximal segment of ventrally located proximal tubules, obtained results from which they conclude that the reabsorption of sugar and chlorides occurs in the first part of the proximal convoluted tubule. Animals injected with large amounts of sugar would thus be expected to show an increase in the relative number of proximal-tubule

TABLE 1

Classification of animals according to treatment and according to the number of globule-filled cells contained in tissue

ANIMALS		NUMBER OF ANIMALS CONTAINING CHARACTERISTIC NUMBER OF GLOBULE-FILLED CELLS		
Total number	Substance injected	Many	Moderate number	Few
14 (1)[1]	Phenol red	13 (1)	0	1
9	Glucose	5	4	0
14 (1)	Urea	13 (1)	1	0
3	Phlorhizin	2	0	1
14 (1)	Phlorhizin and glucose	9 (1)	2	3
37 (3)	Untreated	29 (1)	6 (1)	2 (1)

[1] Figures in parentheses indicate the number of infected animals included in the group.

cells filled with large globules, but, in fact, they exhibit no marked differences in this respect from the normal, or from animals in which reabsorption of sugar is supposedly inhibited by phlorhizin.

The frequent localization of the globule-containing cells at or near the periphery and especially on the ventral side of the kidney, where it is most exposed when the Necturus is opened, suggests that the globules result from modifications or changes due to the effect of exposure and lack of blood supply. It was with the purpose of testing this hypothesis that a comparison was made between tissue fixed immediately and tissue of the same animal fixed after delay. The latter reveals no definite and marked deviations from the former. The number of cells containing large globules appears, in a few instances, to be slightly greater in the tissue with delayed fixation, but the difference is not sufficient to invalidate the interpretation that the phenomena under consideration are normal aspects of kidney structure. The cells of tissue fixed after delay usually appear to be somewhat separated from each other and may take a less brilliant and definite stain than those of tissue removed and fixed immediately after killing and opening the animal, but the constituents of the cytoplasm are essentially the same in both.

Opposed to the interpretation that globules may be formed as a result of exposure to air is the observation that, when Regaud's fluid was injected into the body cavity of the living animal, the kidney contained great or moderate numbers of cells filled with globules. It is concluded that globule-filled cells are normally present in the proximal tubule, for it seems unlikely that postmortem changes of the magnitude required to produce them could occur in the few moments that elapse between pithing an animal and the removal of blocks of kidney.

3. Granules. In addition to globules, smaller spherical bodies of at least two types are distinguished in material prepared by different methods of fixation. They may be described as follows:

a. Small spherical granules. In material fixed by Regaud's and Champy's fluids and stained with acid fuchsin and methyl green, small spherical granules occur which take a green stain. They vary in size, number, and position in the cell. Their distribution in the cell seems to follow no fixed rule, but suggests a relation to the position and number of large red globules. In cells which contain no globules, the small spherical granules may be present in large or small numbers. When numerous, they fill the cell (fig. 4). When less numerous, they may occupy either the supranuclear (fig. 3) or the nuclear region, or both the supranuclear, and in fewer numbers, the nuclear regions (fig. 5). When large red globules are present in the supranuclear part of the cell, the small green granules are found for the most part in the nuclear and basal portions of the cell, the number in the supranuclear region varying inversely with the number of large red globules (figs. 7, 40, 41, and 42). When the cell is entirely filled with globules, the small green granules are found in small numbers in the basal region of the cell (figs. 9 and 10).

The interrelation of number and position of globules and granules is not precisely the same in different animals, but an inverse ratio in numbers is apparent, suggesting the formation of globules from granules. Examples of individual variations are shown in the cells of figures 4 and 8, from one animal, as compared with those in figures 5 and 6, from another. Both show the presence of small green granules and what is considered to be the beginning of the development of large globules. In the latter case, however, the small green granules are less numerous and more confined to one locality than in the former, and evidence of a gradual formation of large globules from green granules is more apparent.

Direct evidence for the genetic relation of the small spherical granules to large globules is lacking, but is further suggested by the gradations of color from the green of the small granules to the red of the large globules. Such gradations in staining reactions might indicate gradual alterations in chemical nature during the transformation, or be due to the size of the granule or globule. It is possible that when the slide is flooded with acid fuchsin, the globules and granules all take the stain; but when the slide is rinsed with water, the smaller granules lose a greater amount of stain in proportion to their size, consequently taking more of the methyl green added after washing.

Material from different animals seems to exhibit slightly different staining reactions, and an entire block of tissue may take and retain more of the red or of the green—possibly due to differences in the tissue itself or in the mordanting of the tissue.

Granules apparently identical with the small green spherical granules just described are found in material fixed by the Mann-Kopsch method, in which they usually have a dark brownish color. Their appearance and distribution are such as to suggest that they first appear in the region of the greatest amount of Golgi material (figs. 21 and 35) and then become dispersed throughout the supranuclear and nuclear regions (figs. 20, 22, and 23), being subsequently pushed basalward and being replaced gradually by large globules (figs. 26 and 36). Direct evidence for a transformation of small granules to large globules is lacking in this material as well as in that prepared for observation of chondriosomes. The small granules are much blacker than the large globules, which sometimes are not darkened at all by the Mann-Kopsch method.

Individual differences in the interrelationships of granules and globules, similar to those found in tissue prepared by the methods of Regaud and Champy, are also found in the material fixed by the Mann-Kopsch method. Examples of variations are shown by drawings. The cells of figures 20 and 23 are taken from the same animal, and show the development and enlargement of small granules in the supranuclear region, and in the latter, the appearance of a few large globules. In the former a slight indication of formation of small granules in the region of Golgi substance is shown in the position marked by an *x*. The drawings of figures 19, 21, and 22 represent cells of another Necturus and show the sequence more definitely.

b. Minute granules. In material fixed by the Mann-Kopsch method (but not by that of Regaud or Champy) minute black granules occur which appear to be identical with the black component of the Golgi substance.[1] Apart from their apparent close association with the Golgi substance, minute black granules are found in either one or both of two conditions in the cell. They may occur in relatively small numbers dispersed throughout the cell; when in greater numbers and concentration, they are arranged in a layer along the distal edge of the cell directly beneath the brush border.

The attachment of these granules to the Golgi substance suggests the possibility that the granules are in the process of formation by or from the latter, but no evidence has been found of their migration from the Golgi substance toward the base of the brush border, where they are most numerous. The granules are usually most numerous and distinct when the brush border is best developed (figs. 34, 35, 36, and 37), and are never present at the distal end of the cell when the brush border is lacking (figs. 20 and 48). Although they are not necessarily found when the brush border is present (figs. 19, 21, 31, and 32), the granules appear to be correlated with it in some way.

4. Crystals. In many Necturus the proximal-tubule cells contain small diamond-shaped crystals which take a very characteristic vivid green stain in material fixed by Champy's and Regaud's mixtures, when stained by the Bensley-Cowdry method (figs. 40, 41, and 42). In tissue fixed by Bouin's method and stained with iron-haematoxylin, they are black. In tissue fixed by the Mann-Kopsch method they are present, but are sometimes distinguished with difficulty from the small spherical granules, since in material so fixed they have an irregular and angular form due to the attachment of very minute pieces of Golgi material (figs. 23, 35, and 36). In some tissue they are black and seem to replace the Golgi material (fig. 43).

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[1] Page 206.

The number of crystals found in material prepared by different fixatives sometimes differs slightly, but a condition is rarely found in which many are shown by one fixative and few by another. Large variations, however, may appear in different animals, so that the tissue of one animal may consistently contain many crystals while that of another contains none. The same variations in the number of crystals are found in both treated and untreated animals (table 2).

5. Vacuoles. Vacuoles occupying three positions are distinguished.

TABLE 2

Classification of animals according to treatment and the relative number of Crystals found in the tissue

ANIMALS		NUMBER OF ANIMALS HAVING TISSUE WITH CRYSTALS			
Total number	Treated with	Many crystals	Moderate number of crystals	Few crystals	None
14 (1)[1]	Phenol red	10[2]	2 (1)	2	1
9	Glucose	5[2]	1	1	2
14 (1)	Urea	6	2	5 (1)	1
4	Phlorhizin	3	0	1	0
13 (1)	Phlorhizin and glucose	6 (1)	1	5	1
38 (3)	Untreated	12	6	14 (2)	6 (1)

[1] Figures in parentheses indicate the number of infected animals included in the group.

[2] Including one animal in which crystals were thought to have been observed, but were difficult to distinguish.

a. A more or less regular row of vacuoles at the distal edge of the proximal-tubule cells of all types and conditions is frequently noted in material prepared by all methods of fixation (figs. 3, 4, 5, 6, 23, 24, 25, 26, 34, 35, 36, and 37).

b. Vacuoles are also sometimes found in the region of the cell above the nucleus in the place usually occupied by large globules. Infrequently, the stained globules occupying this region appear as if inside larger unstained vacuoles (fig. 34). This appearance suggests the possibility that vacuoles of this kind may be spaces formerly occupied by globules which have been dissolved.

c. Vacuoles occupying a third position are sometimes found arranged in an irregular row along the basal edge of the cell (figs. 8 and 23). Chondriosomes in the form of fibrils or granules extend between them.

6. Hyalin body. A homogeneous structure, the hyalin body, is located near the base of the proximal-tubule cells at the side of the nucleus (figs. 6, 19, 21, 27, and 40). It usually takes a red stain when the material is fixed in Regaud's and Champy's fluids, and assumes a light brown color in material prepared by the Mann-Kopsch method. It varies in size and shape. Although the minute black granules found in material fixed by the Mann-Kopsch method are sometimes attached to its edges (fig. 25), no evidence of a consistent relation to granules and globules, chondriosomes, or Golgi material is found, and its function has not been determined.

7. Chondriosomes. The chondriosomes of the proximal-tubule cells may have the form of long or short filaments, or of granules arranged in rows or scattered. The filamentous chondriosomes are very slender, somewhat twisted, and slightly sinuous. They vary in number, depending upon the individual animal and the number of globules present. The variations in number and form of chondriosomes in relation to granules and globules are best illustrated by a series of cells, arranged to represent possible stages in a cyclic process of activity, as follows:

Type 1. The cells of the first of such a series are filled with many chondriosomes, but contain no granules or globules (fig. 1). In tissue with all appearances of being well fixed the chondriosomes are usually the long filamentous type, though sometimes they have the form of short filaments (fig. 2) or rows of granules. If the fixation of the entire tissue is evidently poor, they are usually present in the form of scattered granules. Cells of this first type are of relatively infrequent occurrence, indicating that this stage is quickly passed over, or may be omitted from the cycle. A source of error in the determination of this type of cell is presented in that the small green granules do not show when the tissue is stained very lightly. Such is probably the case in the cell shown in figure 2. If the stain is dark and no granules can be seen, as in figure 1, it is certain that the cell contains none.

Type 2. In the second type of cell, chondriosomes are numerous; the greater number are filamentous, but some are granular in form (figs. 3, 4, and 5). The cell contains small spherical granules which take a green stain in material fixed in Regaud's and Champy's fluids. Crystals may also be present.

Type 3. In the region above and at the sides of the nucleus in cells of type 3, a very few red-staining globules of various gradations in size occur. The chondriosomes are in the form both of filaments and of granules, the latter being present especially in the region of the globules (figs. 6, 7, and 8). Small spherical granules are also present, and cells of some animals contain crystals.

Type 4. In cells of type 4 the chondriosomes show an increased granulation, a few filaments being found at the base of the cell. The number of large globules is greater in the supranuclear region than in type 3, and the small green granules are found mostly in the basal region (fig. 9).

Type 5. The number of chondriosomes is greatly diminished, and in some cells none can be found. The entire cell is filled with large globules of different sizes (fig. 10). Various shades of red characterize the globules, the larger being bright red, the smaller being purplish and sometimes greenish.

If these types of cell represent stages in a cycle, the return to the starting-point may be assumed to occur when the substance of the globules is extruded in some form, probably as a liquid passing through the brush border of the cell. Such a passage has not been definitely observed, though material is sometimes seen in the lumen of a tubule. The return stages would be indicated by a gradual lessening of the number of large globules until the cell is freed of them.

Certain objections to the interpretation that these cells represent stages of activity may be mentioned. It is true that a series such as that described is found in material from single animals or even on single slides. Cells exhibiting different aspects of structure can be found in neighboring tubules or, infrequently, in a single tubule. Differences between individual animals, however, are also striking. The tissue of each individual animal has its own characteristic appearance. Sometimes the chondriosomes throughout the whole tissue are in the form of filaments, or they may be primarily granular throughout; or chondriosomes of both forms may be present in about equal numbers in the greater number of cells. Often correlated with individual differences in the number and form of chondriosomes are individual variations in the number of cells filled with large globules. To account for these individual differences it may be assumed that chondriosomes are actually different in different individual animals, or that the treatment of the tissue produces the different appearances from the same common structure. The chondriosomes show no apparent differences in structure or position due to delayed fixation, although in some cases they seem to take the stain less easily than those of cells in the fixation of which no delay occurred. The preponderance of globule-filled cells in certain regions of sections seems difficult to harmonize with the idea of a cyclic process, unless localization is indication of continued excess activity in some regions and inactivity in others.

By greatly changing the activity of the tubule epithelium by experimental methods such as those described, some stages of the cycle would be expected to be found more frequently than normally, and others less frequently; and it would be expected that individuals treated in a definitely specific way might have chondriosomes definitely modified either in form or number. In animals so treated, however, no one stage or appearance occurs either more or less frequently, and chondriosomes show no marked differences from the normal. In treated animals many or few chondriosomes may be present; and the forms of the chondriosomes may be any or all of those found in normal animals. The number of animals showing these conditions is given in table 3.

6. Golgi substance. *a.* In untreated animals. The Golgi material of the cells of the proximal tubule of untreated Necturus varies in form. Its apparent structure differs with the extent to which differentiation of the osmium is carried by potassium permanganate. When slightly differentiated, the Golgi bodies appear as irregular homogeneous black masses or filaments of irregular outline (fig. 22). With further dif-

TABLE 3

Classification of animals according to treatment and according to the number and form of chondriosomes contained in the tissue

ANIMALS		NUMBER OF ANIMALS SHOWING CHARACTERISTIC CHONDRIOSOMES							
		As to number		As to form					
Total number	Substances injected	Many	Few	Scattered granules	Filaments	Mostly granules, some filaments	Equal number of filaments and granules	Granules in rows	Chondriosomes indistinguishable (poor fixation)
				Regaud's method					
12 (1)[1]	Phenol red	3	7 (1)	4	4	4	3	2	5 (1)
6	Glucose	3	0	2	1	3	4	3	0
12 (1)	Urea	4 (1)	4	4 (1)	3	3	6 (1)	2 (1)	2
2	Phlorhizin	0	0	1	2	0	1	0	0
11 (1)	Phlorhizin and glucose	2 (1)	3	6	5	3 (1)	5	1 (1)	2
34 (2)	Untreated	6	11	13 (1)	11 (1)	9	10 (2)	13	5
				Champy's method					
9	Phenol red	0	2	4	0	3	4	0	3
3	Glucose	1	1	3	1	1	2	1	0
9	Urea	0	3	2	2	1	1	0	3
2	Phlorhizin	0	2	1	0	2	2	0	0
10	Phlorhizin and glucose	2	2	5	1	4	4	1	2
19 (1)	Untreated	1	11	9 (1)	5 (1)	3	8 (1)	3	3

[1] Figures in parentheses indicate the number of infected animals included in the group.

ferentiation each filament may be seen to consist of a pale-gray ground-substance, irregularly covered with minute black granules (figs. 19, 20, and 21). The substance may be scattered through the cell in fragments of irregular shape and size (figs. 25 and 27).

The position of the Golgi bodies in the proximal-convoluted-tubule cell is nearly constantly that of the region surrounding the equator of the nucleus (pl. 4), but it may occur slightly nearer the lumen side (figs. 23, 24, and 25) and, infrequently, nearer the basal side of the cell. In one animal (no. 147) the position of the Golgi substance was definitely supranuclear.

As in the case of chondriosomes, a series of cells exhibiting different aspects of the Golgi material perhaps representing stages of cyclic activity may be arranged.

Type 1. The first of the series contains a large amount of Golgi material in the form of long threads of ground-substance with small black granules attached. No granules or globules are present (figs. 19 and 34).

Type 2. The second type contains abundant Golgi substance in filamentous strands, and is characterized by the presence of small spherical granules which, in some cells, appear only in the region of the Golgi material (fig. 21) and, in others, fill the nuclear and supranuclear regions (fig. 22). They are evidently to be identified with the small green spherical granules of material fixed by the Regaud and Champy methods. Crystals may also be present (fig. 35).

Type 3. In the third type the Golgi material is usually partly broken into fragments, and large globules appear in the supranuclear region of the cell and in the nuclear region toward the lumen side (figs. 23, 24, and 36).

Type 4. As the number of globules increases in the supranuclear region, the Golgi material is scattered throughout the cell in fragments which are frequently entirely black (fig. 25). The cell may also contain small spherical granules (fig. 26) which are more darkly stained than the globules and are located at the sides of the nucleus in the region of the greatest amount of Golgi material.

Type 5. The Golgi material is lessened in amount and is scattered through the cell, which is entirely filled with globules (figs. 25, 27, and 37).

Although such a series can be found in some animals (pl. 7), each individual has its own characteristic Golgi formations. The entire tissue of one animal may contain Golgi substance in no form other than scattered fragments (figs. 32 and 33), and the tissue from another may contain the filamentous type almost exclusively (pl. 7). It is especially noticeable that the Golgi material is very frequently similar in form in proximal- and distal-tubule cells of the same individual (compare figs. 26 and 27 with figs. 28 and 29, also fig. 30 with fig. 31 and fig. 32 with fig. 33; see also pl. 7). This suggests that the tissue of each individual animal has its own characteristic physicochemical constitution, causing these appearances, or that the fixative produces these characteristic effects from the same typical structure because of some unknown, uncontrolled factor in the method of fixation. The Golgi substance in two cases of delayed fixation seemed to be slightly increased in degree of impregnation, while in others it seemed to be decreased in amount in comparison with that of tissue fixed without delay. It appears that the necessary short interval occurring between the beginning and the end of the process of taking kidney tissue for fixation probably does not affect the Golgi substance, and that differences in form of Golgi substance cannot be attributed to effect of exposure and lack of blood supply.

b. In treated animals. The variations in form and position of Golgi substance in both proximal- and distal-tubule cells of treated animals are of the same nature and magnitude as found in untreated animals, and, therefore, show no definite correlation with the physiological processes induced in these experiments (table 4).

A suggestion of an effect of treatment was found, however, in tissue of animals no. 132, 134, 139, and perhaps 133, in the proximal tubules of which the cell constituents impregnated with osmium have an appearance noticeably different from that observed in tissue of any other animals. Since these animals were all injected with large amounts of sugar, this difference may be an indication of a change correlated with increased reabsorption of sugar. The Golgi substance consists of light gray and black rods and indefinitely shaped bodies (figs. 44, 45, and 46). It appears as if this aspect of the Golgi substance might result from its association with numerous diamond-shaped crystals, since by other methods of fixation (Regaud and Champy) the tissue of all these animals showing this peculiarity contains crystals. The condition exhibited by the osmiophilic substance of the tissue of animals injected with sugar, however, is distinguishable from that observed in tissue of other animals in which large numbers of crystals are present (figs. 35, 36, and 43).

TABLE 4

Classification of animals according to treatment and according to the amount and form of Golgi substance contained in the proximal-tubule cells

ANIMALS		NUMBER OF ANIMALS HAVING CHARACTERISTIC GOLGI SUBSTANCE									
		As to amount			As to form						
Total number	Substances injected	Large	Moderate	Small	Filaments	Fragments and some filaments	Fragments	Filaments and some fragments	Granules replacing Golgi bodies	Crystals replacing Golgi bodies	Golgi bodies absent (poor fixation)
9 (1)[1]	Phenol red	1	3	5 (1)	0	4 (1)	4 (1)	2	2	4	1
7	Glucose	1	0	5	1	2	2	2	5	4	0
9	Urea	0	2	6	0	6	4	1	3	4	1
3	Phlorhizin	0	2	1	0	1	0	2	0	1	0
7 (1)	Phlorhizin and glucose	0	2	4 (1)	0	3 (1)	1	0	1	2 (1)	1
30 (3)	Untreated	8 (1)	1	13	10 (1)	4 (1)	8 (1)	10 (1)	6	1	8 (1)

[1] Figures in parentheses indicate the number of infected animals included in the group.

The appearance of Golgi substance in the tissue of the other two Necturus, which were injected with large amounts of sugar (no. 138 and no. 140), is not similar to those just described, and yet differs somewhat from that of animals not injected with sugar. In the tissue of animal no. 138 the cells are filled with numerous small black granules that are of slightly different sizes in different cells (fig. 48). Similar black granules are present in smaller numbers in the tissue of animal no. 140. By comparison with material from the same animals fixed in Regaud's fluid, it is found that the cells of animal no. 138, but not of no. 140, contain diamond-shaped crystals. This condition is less markedly characteristic for animals injected with sugar, since the tissue of two normal animals (no. 61 and no. 147) shows somewhat similar structures (fig. 32). Cells of animal no. 147 contain also a few very short rod-shaped Golgi fragments slightly suggestive of the large rods found in cells of animals no. 132, 133, 134, 139.

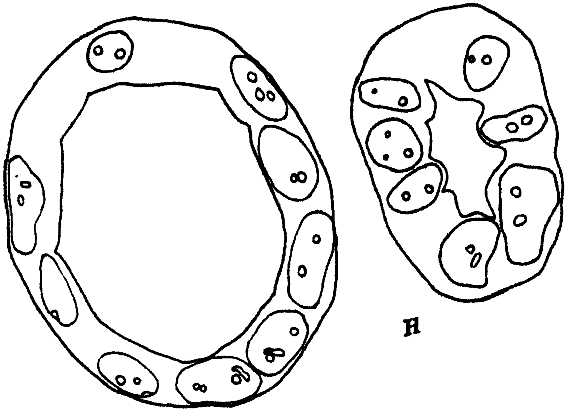
Since the tissue of animals that received one injection only of glucose (animals no. 8, 9, 51) shows no difference from that of untreated animals, the observations suggest that changes may occur in the osmiophilic constituents of the cell, provided very large amounts of glucose are injected.

III. Distal-tubule cells

A. Shape and size. Cells that constitute the epithelium of distal segments are smaller in size than those of the proximal segment. They are characteristically low, and the side nearest the lumen of the tubule is usually concave, though in some cases it may slightly protrude (pl. 3; text figs. G, H). Distal cells show wide variations in size that are associated with correspondingly wide variations in the diameter of the tubule and of the lumen (figs. 12, 14, 28, and 29).

No correlation is found between diversities in size of these cells and the observed differences in their structural elements.

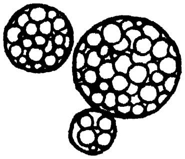
B. Constituents. 1. Chondriosomes. Though the chondriosomes of the distal tubules show some variations, they are more constant in form than those of the proximal-tubule cells. They sometimes have the form of 'Stäbchen,' or of thick rods, but their apparent thickness is probably due to an excess amount of stain that is held by a closely packed group of chondriosomes (figs. 12 and 14), for with lighter stain the chondriosomes appear as slender filaments. They extend from the base of the cell to a clear border of cytoplasm at the distal edge (figs. 15 and 16). The chondriosomes usually are distributed uniformly throughout the cytoplasm, but, in some cases, the filaments are somewhat grouped. In some cells the chondriosomes are granular in form (fig. 17). This latter condition occurs more frequently, but not exclusively, in parts of blocks of the kidney which have the general appearance of poor fixation. Regaud's mixture almost invariably produces excellent fixation. Of seventy-five blocks of material taken from untreated and treated animals and prepared by this method, fifty-six uniformly contained typical filamentous chondriosomes. In the tissue of thirteen animals the fixation was somewhat faulty, some regions containing filamentous chondriosomes and other regions, evidently poorly fixed,



G

H

Text figs. G and H Outlines made with camera lucida of cross-sections of distal tubules, showing variations in size. × 680.



I

Text fig. I Camera-lucida drawing of large globules made up of small globules. × 680.

containing granular and grouped chondriosomes. No blocks were poorly fixed throughout. This consistency in the form of chondriosomes in the distal tubules, after fixation by Regaud's fluid, is striking.

In material fixed in Champy's fluid the chondriosomes are less well fixed. Of fifty-four blocks of tissue, only five consistently contained filamentous chondriosomes; twenty-eight were distinctly poorly fixed, and in twenty-two the fixation might be described as fair. In twenty-seven of the last two groups the material was taken from the same animals in which good results were obtained by fixation with Regaud's fluid. This difference in result finds an explanation in the fact that the constituents of Champy's fluid penetrate less rapidly than those of Regaud's fluid, and that the distal tubules are located in the interior of the kidney. It is concluded that the chondriosomes of the cells of the distal tubules constantly maintain the form of filaments. When the chondriosomes, in otherwise excellently fixed blocks of tissue, consist of rows of minute granules, it may be that in certain individual animals this form is normal, or, if due to artifact, that chondriosomes are the least stable elements of the cell and hence the first to undergo postmortem or fixation disorganization (fig. 17).

The same variations in chondriosomes occur in the distal-tubule cells of treated and untreated animals, with no evident difference in frequency (table 5).

2. Golgi substance. The Golgi substance in the distal-tubule cells is somewhat similar to that in the proximal-tubule cells, in that it may consist of small black fragments or of a somewhat filamentous, light ground-substance with adhering small black granules (figs. 13, 30, and 31). The filamentous form is usually, but not always, shorter and coarser than that of the proximal-tubule cells (figs. 18, 28, and 29). As has been pointed out, the form of the Golgi substance is frequently, but not universally, similar in the proximal and distal tubules of the same individual animal.[2]

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[2] Page 209.

The Golgi substance shows greater variations in position in distal- than in proximal-tubule cells. In the former it is often located near the edge of the cell near the lumen (figs. 13, 28, and 29). The position most frequently occupied is that of the region between the nucleus and the lumen. In some blocks of tissue the Golgi substance is not constant in position, being located definitely in the supranuclear region of some cells and in the nuclear region of others (figs. 38 and 39). In other individuals the Golgi substance may be found consistently nuclear in position (figs. 18 and 30). In one animal only (no. 87), the position of Golgi bodies in a number of sections of tubules was found to be below the nucleus. Tables 6 and 7 classify the animals according to the form and position of Golgi substance in the distal-tubule cells.

TABLE 5

Classification of animals according to treatment and according to the form in which chondriosomes occur in the distal-tubule cells

		NUMBER OF ANIMALS SHOWING CHARACTERISTIC FORM OF CHONDRIOSOMES					
Total number	Substance injected	Filamentous		Filamentous and granular		Poor fixation	
		Champy	Regaud	Champy	Regaud	Champy	Regaud
14 (1)[1]	Phenol red	1	8 (1)	4	3	2	0
9	Glucose	0	6	2	1	1	0
14 (1)	Urea	0	9 (1)	3	1	7	0
3	Phlorhizin	0	1	1	1	1	0
13 (1)	Phlorhizin and glucose	0	8 (1)	5	2	3	0
42 (2)	Untreated	3	26 (1)	7	7 (1)	14 (1)	6

[1] Figures in parentheses indicate the number of infected animals included in the group.

IV. *Tissue of infected animals*

Tissue taken from infected animals exhibits no conditions not found in normal animals. The number of infected animals studied is not sufficiently large to afford a basis for definite conclusions concerning the effect upon the kidney of infection of the body cavity, but, since no abnormality in cell structure is found, there is no apparent reason for excluding them from a record of results of experiments. The figures in parentheses in the tabulations of results indicate the number of infected animals in the record.

TABLE 6

Classification of animals according to treatment and the characteristic form of Golgi substance in distal tubules

ANIMALS		NUMBER OF ANIMALS SHOWING CHARACTERISTIC FORM OF GOLGI SUBSTANCE IN THE DISTAL-TUBULE CELLS			
Total number	Substance injected	Filaments	Mostly granules, some filaments	Fragments	Mostly filaments, some granules
9 (1)	Phenol red	2	2	1	2
7	Glucose	5	2	1	4
8	Urea	6	0	0	2
3	Phlorhizin	2	1	1	1
7 (1)	Phlorhizin and glucose	1	2 (1)	2	1
30 (2)	Untreated	12 (2)			

TABLE 7

Classification of animals according to treatment and the characteristic position of Golgi substance in distal tubules

ANIMALS		NUMBER OF ANIMALS SHOWING CHARACTERISTIC POSITION OF GOLGI SUBSTANCE IN DISTAL TUBULES			
Total number	Substance injected	Nuclear	Supranuclear	Both	Upper nuclear
5 (1)[1]	Phenol red	2 (1)	2	1	0
6	Glucose	0	3	2	1
7	Urea	2	2	1	2
3	Phlorhizin	1	0	1	1
5 (1)	Phlorhizin and glucose	1	1	3 (1)	0
23 (2)	Untreated	4 (1)	4	12 (1)	3

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[1] Figures in parentheses indicate the number of infected animals included in the group.

SUMMARY OF OBSERVATIONS

The principal observations of this cytological study of the mesonephros of Necturus may be briefly stated as follows:

I. Chondriosomes

A. Proximal-convoluted-tubule cells. 1. Chondriosomes may occur primarily in the form of long fibrils, or of fibrils and granules in the same cell, or of granules arranged in rows or irregularly scattered throughout the cell. Variations in form have not been demonstrated to be correlated with physiological activities which have been induced by experimental treatment.

2. In treated and untreated animals cells occur that may be arranged in a series illustrating an inverse ratio between the number of chondriosomes and the number of large globules present. Material from different animals, however, often exhibits characteristic appearances which do not conform with the interpretation that such a series represents stages in a cyclic process of activity.

3. When tissue contains primarily granular chondriosomes throughout, the form may be due to changes from the living state due to manipulation or fixation, to unexplained individual differences, or to physiological states not controlled in these experiments.

B. Distal-tubule cells. 1. Chondriosomes of the distal tubules appear in the form of thick rods when heavily stained, but are observed to be slender filaments when a lighter stain is obtained.

2. Cells with granular chondriosomes are usually obviously poorly fixed; but cells that are apparently excellently preserved are found in which the granular form occurs, indicating perhaps a first stage of cell injury or artifact.

3. The same variations in form of chondriosomes occur in treated and untreated animals.

II. Golgi material

A. Proximal-tubule cells. 1. Golgi substance is found in the form of filaments to which minute black granules adhere, or in the form of scattered black fragments. The same variations in form occur in treated and untreated animals.

2. Golgi substance in tissue from some Necturus injected with glucose shows a characteristic appearance of large black or gray rods and indefinitely shaped bodies which suggests correlation with the process of reabsorption of sugar by the proximal-tubule cells.

3. As in the case of chondriosomes, a series of cells may be arranged showing a graded decrease of Golgi substance and a dispersal of Golgi fragments with increased number of globules. In treated and untreated animals, however, individual differences in the form and amount of Golgi substance occur which may make invalid the interpretation of such a series as stages in a cyclic process of cell metabolism.

4. The Golgi material is almost uniformly located in the nuclear or the upper nuclear region of the cell.

B. Distal-tubule cells. 1. Golgi material appears in the form of filaments to which black granules are attached, or consists of scattered fragments.

2. The position of the Golgi substance varies more than in the proximal-tubule cells, but has not been found to vary in correlation with any condition induced by the experimental procedures employed. Golgi substance is most frequently found in the supranuclear region.

III. Other aspects and constituents of proximal-tubule cells

A. Vacuoles. Vacuoles have been observed in three locations in the proximal-tubule cells.

1. A regular row of large vacuoles occurs in the area just beneath the brush border and basal granules. This is present in nearly all proximal-tubule cells.

2. Scattered vacuoles may occur in the supranuclear region of the cell. These probably represent spaces formerly occupied by granules that have been dissolved.

3. Vacuoles occupying a third position are sometimes found in an irregular row at the basal border of the cell.

B. Brush border. A striated brush border is probably constantly present in normally functioning proximal-tubule cells, although cells occur in which striations do not appear, and others lack it entirely.

C. Abnormal cells of the proximal tubule. Cells of the proximal tubule are described which have no brush border, contain lightly staining cytoplasm, and usually contain no granules and little or no chondriosomes or Golgi material. These cells may extend over and obliterate the brush border of neighboring normal cells, and some apparently become squeezed out and extruded into the lumen. They are interpreted to be abnormal or perhaps necrotic.

D. Minute black granules. Minute black granules, apparently identical with the black component of the Golgi substance, are found scattered in the cells of tissue prepared by the Mann-Kopsch method of fixation and concentrated at the distal end of the cell directly beneath the brush border.

E. Crystals. Diamond-shaped crystals are often found in cells of the proximal tubule. Their nature has not been determined. They are found in material fixed by the methods of Regaud, Champy, Bouin, and Mann-Kopsch. The number of crystals is not altered by the experimental treatments of Necturus carried out in this investigation.

F. Globules. Many or few large cells filled with globules may be contained in the kidney. The largest cells containing the largest globules are usually located at and near the periphery of the block of tissue, and are usually more numerous on the ventral than on the dorsal side of the kidney. This condition is probably not due to exposure of the tissue, but may be indicative of activity of primary tubules and of the part of the secondary tubules following Bowman's capsule, which courses toward the periphery. The treated animals show no marked differences from the untreated in the number of globule-filled cells.

G. Small spherical granules. Small spherical granules in proximal-tubule cells may represent early stages in the development of large globules.

DISCUSSION

The cytological study of the mesonephros of Necturus has not demonstrated definite changes in the form, amount, or position of chondriosomes and Golgi material which can be interpreted with certainty to be correlated with increased secretion of phenol red and urea, or with increased or decreased reabsorption of glucose. Variations in the cell constituents occur in material from untreated as well as treated Necturus in tissue which has every appearance of being normal and successfully fixed.

Correlation of variations in the position of Golgi material with secretion and reabsorption, such as have been described by Jasswoin ('25), Avel ('24), and Basile ('15), has not been found. Both in normal animals and in those with modified secretion or reabsorption induced by the methods used in this investigation, the Golgi material is nearly constantly located in the nuclear region of the cells of the proximal convoluted tubule, and usually, but less uniformly, supranuclear in the cells of the distal tubule. Variations in position are evidently either constant individual differences or are correlated with metabolic changes not controlled in these experiments. These observations confirm the conclusions of Nassonov ('27), who considers that variations in the position of the Golgi material are individual differences, not determined by the polarity of cell activity, and of Turchini ('27), who concludes that polarity of cells cannot be determined by the position of the Golgi substance.

When the Golgi substance assumes a form other than that of threads with adhering black granules which might be supposed to be typical, it is important to determine with certainty whether these supposedly atypical forms are due to changes involved in the physiological activity of the Golgi material as a cell organ, or due purely to fixation effects.

If the tissue is manifestly poorly fixed, variations in form and amount may obviously be attributed to faulty fixation; but when in well fixed tissue the Golgi substance shows variations, the inference is warranted that it probably assumes different forms in the living cell.

The tissue of animals injected with glucose is the only material in which the Golgi substance is somewhat characteristically different from that of animals not so treated and, therefore, appears to undergo changes apparently correlated with the process of reabsorption of sugar. In all other animals many variations in form and amount of Golgi material occur which are evidently not definitely correlated with the increased excretion of the substances injected into the living animal, since they appear in untreated as well as in treated animals.

The results of this investigation do not definitely prove or disprove the conclusions of Walker and Allen ('27) that the Golgi bodies, so characteristic of animal cells, are artifacts resulting from coagulation of certain ergastic lipoid substances during fixation, and are not to be regarded as cell organs. The numerous variations in form assumed by the Golgi substance of the kidney cells of Necturus might be considered support for such a theory; but, at the same time, the regularity of the form does not justify the abandonment of the idea that this lipoid substance represents a structure of cytoplasmic organization. The regularity with which characteristic forms appear in different kinds of cells is an aspect not obtained in artificial cells.

Close relationship between the position of Golgi substance and that of granules and globules is not always found. The presence in the proximal-tubule cells of globules and granules apparently somewhat different in nature suggests a similar condition described by Nassonov ('27) in epididymal cells; but, in the proximal-tubule cells of the kidney of Necturus, no evidence is found for the distinct origin of both in the region of the Golgi substance, or of migration of one kind to the base of the cell and of the other to the lumen, each remaining separate. The small granules usually seem to appear first in the region occupied by the greatest amount of Golgi material—which suggests the interpretation that they take origin from, or under the influence of, Golgi substance; but cells occur in which this close topographical relationship is not evident, though a migration of the granules from the Golgi substance in these cases may have already taken place (figs. 20 and 24). The globules increase in number first in the supranuclear region, and later fill the whole cell. They do not appear to have a definite relation to the Golgi substance during their formation.

The determination of the effect of fixatives upon the chondriosomes is also important in the interpretation of changes which might be attributed to physiological activity. The chondriosomes of the distal tubules are nearly constant in form, but those of the proximal-tubule cells are found to vary considerably in tissue which is certainly excellently fixed. Cowdry ('18) warns that interpretations based upon changes in the morphology of chondriosomes must be made with great care, since these structures are evidently the constituents of the cell that are most sensitive to injury. Certain appearances in the tissue used in this investigation, as of extreme granulation and, in some cases, absence of chondriosomes, are readily seen to be due to poor fixation or injury, the latter being evident at cut edges of the block of kidney. The cause of poor fixation, however, is unknown, and might have some relation to the physiological state of the animal. When material is well fixed, marked differences in the form of chondriosomes of different animals are attributed to unexplained individual characteristics, or to changes occurring in correlation with metabolic activities other than those induced by the methods employed in this investigation. No correlation between variations in the number and form of chondriosomes and the physiological conditions induced has been found. The results seem to confirm the conclusion of Policard ('10) that, whether the kidney is active or inactive, the chondriosomes of the proximal convoluted tubule of normal animals undergo modifications of the same amplitude. Policard finds no relationship in the kidney of the frog between the number of granules and the number of chondriosomes, such as has been found between globules and chondriosomes in the Necturus kidney. Regaud ('09), however, describes an inverse relationship in the kidney of reptiles and Amphibia between the number of chondriosomes and globules, similar to that described in this study, and considers the observation evidence that globules form from chondriosomes. It is observed in this investigation that, in cells filled with large globules, both the Golgi substance and the chondriosomes are decreased in amount. This suggests that both chondriosomes and Golgi material contribute to the formation of globules. Ma ('28) finds evidence that zymogen granules of the cells of the stomach and pancreas form in relation to both chondriosomes and Golgi material.

Some investigators have found evidence that the number of large globules increases under certain conditions. Barratt ('13) describes a great increase in number of globules correlated with increased activity of the kidney during hemoglobinemia; and Thomas and Dragoiu ('27) find an increase in number of globules in the kidney of young guinea-pigs when large amounts of pentose were ingested. The suggestion has also been made that they may result from exposure of the kidney. If the preponderance of globule-filled cells that often characterizes the proximal tubules of both treated and untreated animals is not a normal state of the functioning tubule, but is due to postmortem changes, the globules must form immediately after the animal is pithed; for, as is also the case with chondriosomes and Golgi bodies, their number is not altered between early and delayed fixation.[3] If modifications great enough to transform the normal constituents of the proximal-tubule cell into a mass of globules do occur in Necturus as the result of pithing and exposing the kidney, interpretations concerning normal function of living organs are hardly valid when based upon results of physiological experimentation in which organs and tissues are subjected to injury much greater and of longer duration than is involved in removing tissue for fixation.

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[3] Page 199.

The apparent lack of correlation between induced physiological processes and changes in the cytoplasmic constituents of Necturus kidney might be explained by the assumption that the kidneys of many of the untreated Necturus were already so active that those with supposedly induced activity showed no marked differences from the normal, the structural mechanisms for the transfer of the injected substances being the same as those normally present. If the presence of numerous large globules can be used as a criterion of extreme activity, however, such a hypothesis is invalidated by the fact that the tissue of some animals treated to increase activity contains but few globules.

SUMMARY

A cytological study of the mesonephros of Necturus maculosus has been made to determine whether structural elements of the cytoplasm can be demonstrated to be specifically concerned with certain physiological processes that are experimentally induced in the tissue at the time of fixation. The methods for bringing into view variations in cell structure correlated with cell activity are as follows: the increase of the process of secretion by subcutaneous injection into the living animal of phenol red and of urea; the increase of the process of reabsorption by injection of glucose; the inhibition of reabsorption by injection of phlorhizin.

Variations of the same magnitude in form, position, quantity, and interrelationships of granules, globules, chondriosomes, and Golgi substance were found to occur in normal, untreated animals and in those in which specific physiological processes were sought to be experimentally induced. Some evidence, however, is found that in cells of the proximal tubule a characteristic form of Golgi body appears which seems to be correlated with increased reabsorption of sugar.

These observations suggest that the kidney cells of normal Necturus are constantly so active that correlated rhythmic changes, occurring normally in the structural elements of the cell, are not significantly altered when other substances also are presented for transportation through the cells.

Although experimental treatments thus far given have failed to induce variations in the structural elements of proximal or distal cells of sufficient magnitude to demonstrate clearly correlations with specific physiological processes, it does not necessarily follow that proof is thereby afforded that no such correlation exists. The results indicate that the methods employed for revealing this correlation are inadequate and that other methods must be tried. Further work is in progress in which relatively very large quantities of glucose are injected, with the aim of modifying structural elements correlated with the sugar-reabsorption process.

The great diversity in amount, form, and interrelationships of the structures observed in cells of the proximal tubule, the characteristic constancy of structures present in cells of the distal tubule, and the general uniformity with which these configurations occur in corresponding cells of the kidney of vertebrates other than Necturus seem difficult to harmonize with an interpretation that the several structural elements of these cells have no specific vital function to perform in the processes of excretion, but are to be regarded either as artifacts or as visible ergastic substances.

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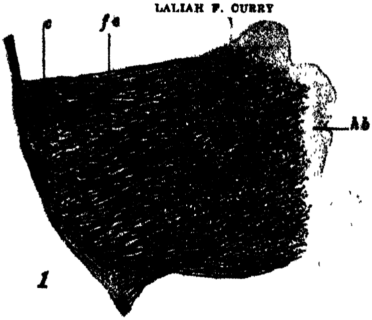

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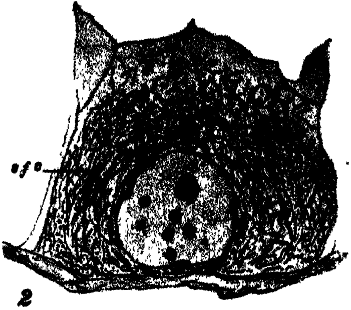

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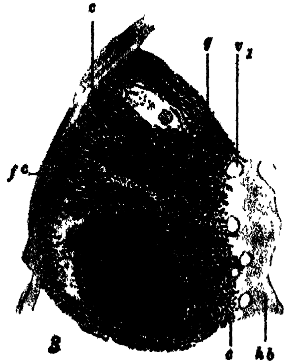

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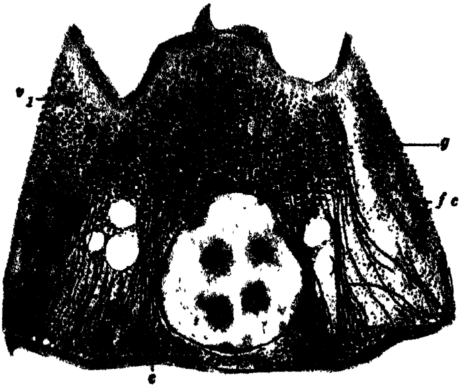

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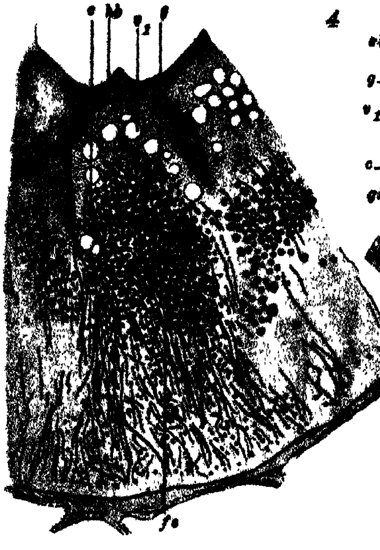

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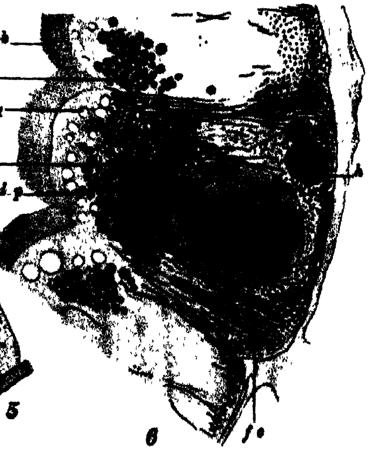


6

EXPLANATION OF PLATES

All plate figures are drawn with camera lucida to the same scale of magnification (1830 diameters) and represent parts of tubules in exact transverse section.

Drawings for which the number of the individual is not indicated were made from material prepared by Doctor Grave before this particular investigation was undertaken.

ABBREVIATIONS

a, abnormal proximal-tubule cell
bg, minute black granules at base of brush border
sb, brush border evenly striated
hb, homogeneous border
cs, crystals
fc, filamentous chondriosomes
c, granular chondriosomes
sfc, short filamentous chondriosomes
g, small spherical granules (green in material fixed by Regaud's and Champy's fluids; dark brown in material fixed by the Mann-Kopsch method)
gl, globules
gl r, globules taking a red stain
gl p, globules taking a purplish or greenish stain
G, Golgi substance
Gs, ground-substance of Golgi material
sbg, minute black granules showing in Golgi preparations
Gf, Golgi substance in the form of filamentous strands
h hyalin body
n, nucleus
v_1, v_2, v_3, vacuoles of types 1, 2, 3, respectively
w, cell wall

PLATE 1

EXPLANATION OF FIGURES

All drawings represent cells of the proximal tubule. Drawings 1 to 10 illustrate the series described in the text.

1 Numerous long filamentous chondriosomes; no small granules. Brush border homogeneous. (Necturus no. 5; Regaud.)

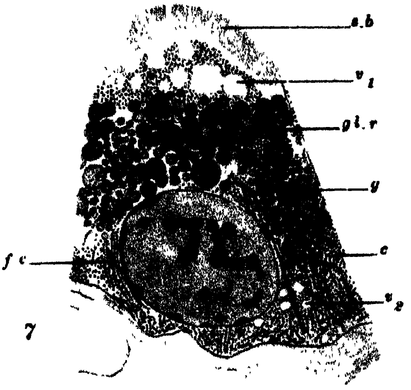
2 Short filamentous chondriosomes; no small granules; cell stained lightly. Brush border homogeneous. (Necturus no. 5; Regaud.)

3 Very small green spherical granules; numerous filamentous and some granular chondriosomes. Brush border homogeneous. (Necturus no. 59; Regaud.)

4 Many small green spherical granules; chondriosomes predominantly filamentous. (Regaud.)

5 Chondriosomes few in number; some are fragmentary; green granules are slightly larger. Brush border homogeneous. (Necturus no. 120; Regaud.)

6 Larger granules that stain with a reddish tinge. Brush border clearly and evenly striated. (From same slide as fig. 5.)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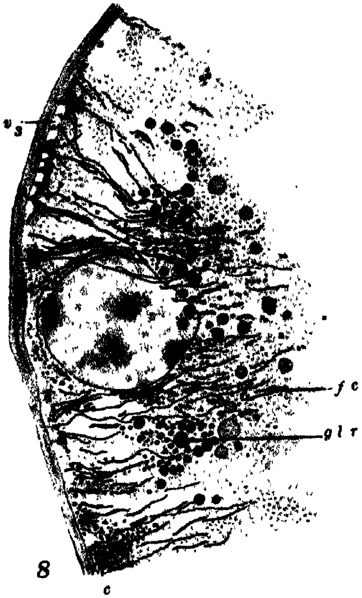
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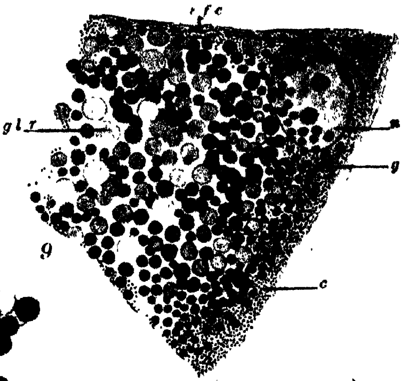
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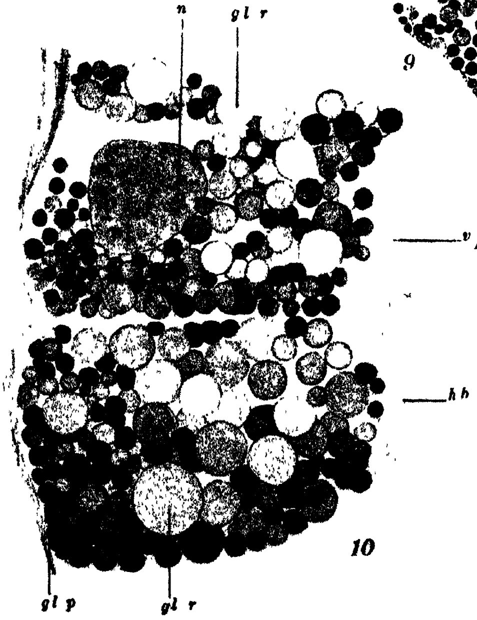
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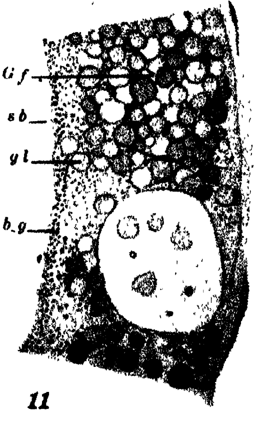
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PLATE 2

EXPLANATION OF FIGURES

All drawings represent cells of proximal tubule.

7 Small green granules; supranuclear region containing globules of various sizes, the larger staining a clear red, the smaller staining purplish and greenish. (Necturus no. 40; Regaud.)

8 Cytoplasm filled with small light green granules, and a few scattered globules. Brush border nearly all absent. (Regaud.)

9 Cell nearly filled with medium-sized globules. At base of cell the small globules stain green. Chondriosomes few in number and fragmentary. Indication of homogeneous brush border at distal edge. (Necturus no. 66; Regaud.)

10 Typical large globule-containing cell. Gradations of shades show the variations in stain apparently correlated with size of the globules. (Necturus no. 112; Regaud.)

11 Very low cell containing many globules. (Necturus no. 52; Mann-Kopsch.)

PLATE 3

EXPLANATION OF FIGURES

All drawings represent cells of the distal convoluted portion of the tubule.

12 Large cells; thick homogeneous chondriosomes. (Regaud.)

13 Large cells; Golgi material in form of filaments with adhering black granules; located in supranuclear position. (Necturus injected with phlorhizin; Mann-Kopsch.)

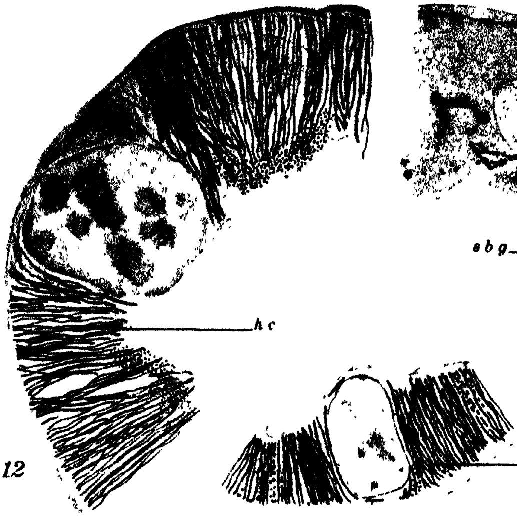
14 Very small cell; thick chondriosomes. (Necturus no. 46; Regaud.)

15 Medium-sized cell; slender filamentous chondriosomes. (Necturus no. 75.)

16 Tubule with high cells, small lumen. Filamentous chondriosomes with lighter stain; some granular chondriosomes. (Necturus no. 40; Regaud.)

17 Cell from same animal as figure 14; predominantly granular chondriosomes.

18 Large cells with Golgi substance in nuclear position. (Normal Necturus; Mann-Kopsch.)



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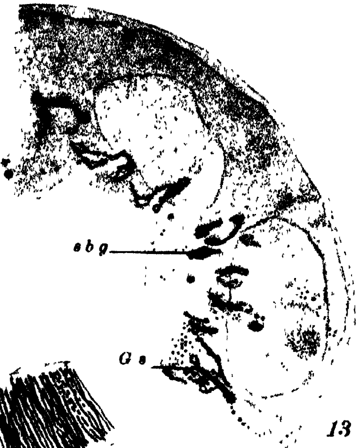

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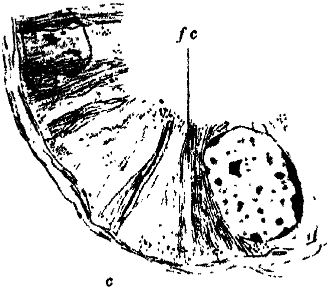

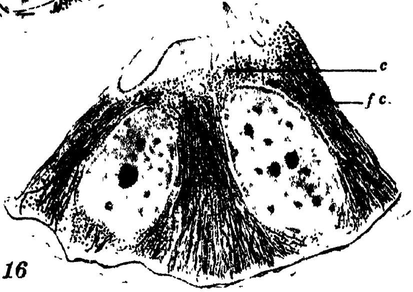

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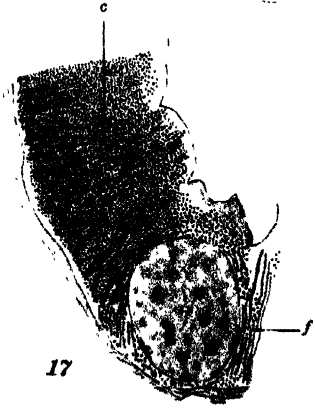

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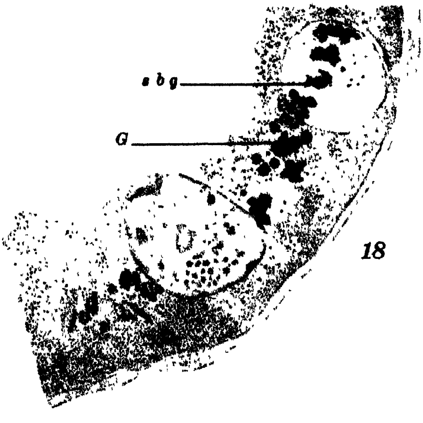

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PLATE 4

EXPLANATION OF FIGURES

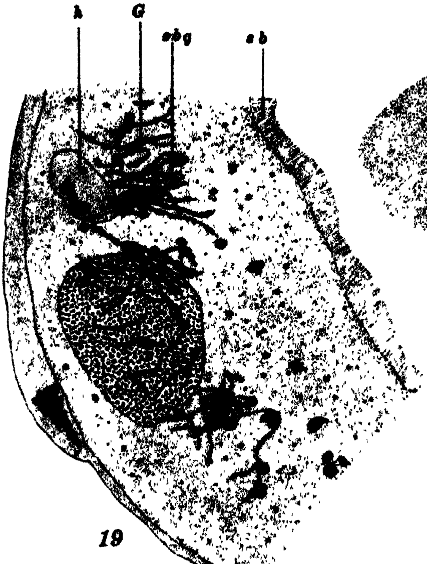
All drawings represent proximal-tubule cells fixed by the Mann-Kopsch method. Drawings 19 to 27 illustrate the series discussed in the text.

19 Abundant Golgi material in form of threads with adhering small black granules. No granules visible in cytoplasm. (Necturus no. 7.)

20 Abundant filamentous Golgi bodies with adhering small black granules. Very small granules lightly stained in supranuclear region and, to a smaller extent, in region of the Golgi bodies

21 Cell from same slide as that represented by figure 19, showing small granules which are evidently identical with the small green granules found in tissue fixed in Regaud's fluid, in region of the Golgi substance. (Necturus no. 7.)

22 Cell from same slide as those represented by figures 19 and 21; small granules, very numerous, filling supranuclear and upper nuclear regions. Golgi substance slightly smaller in amount than in figure 21. Differentiation between light ground-substance and black granules not apparent.



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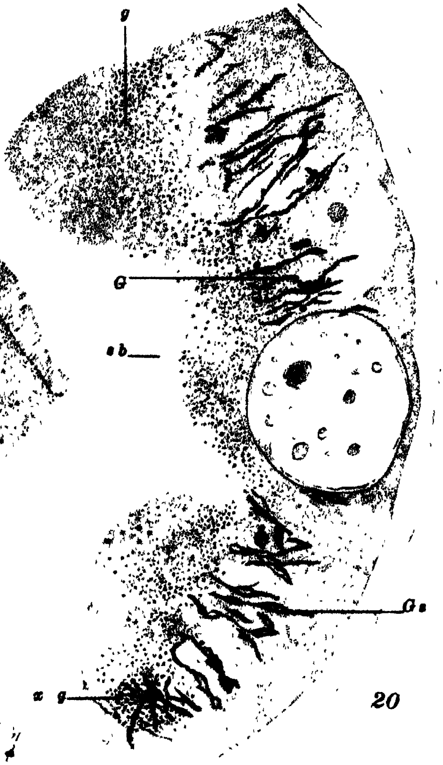

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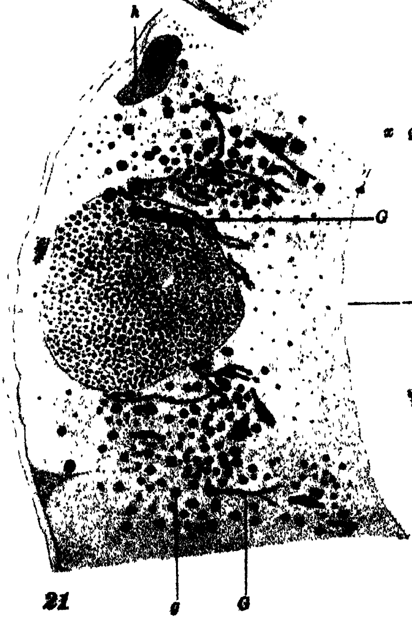

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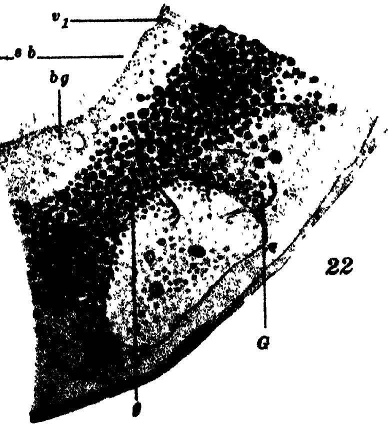

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PLATE 5

EXPLANATION OF FIGURES

All drawings represent proximal-convoluted tubule cells, fixed by the Mann-Kopsch method.

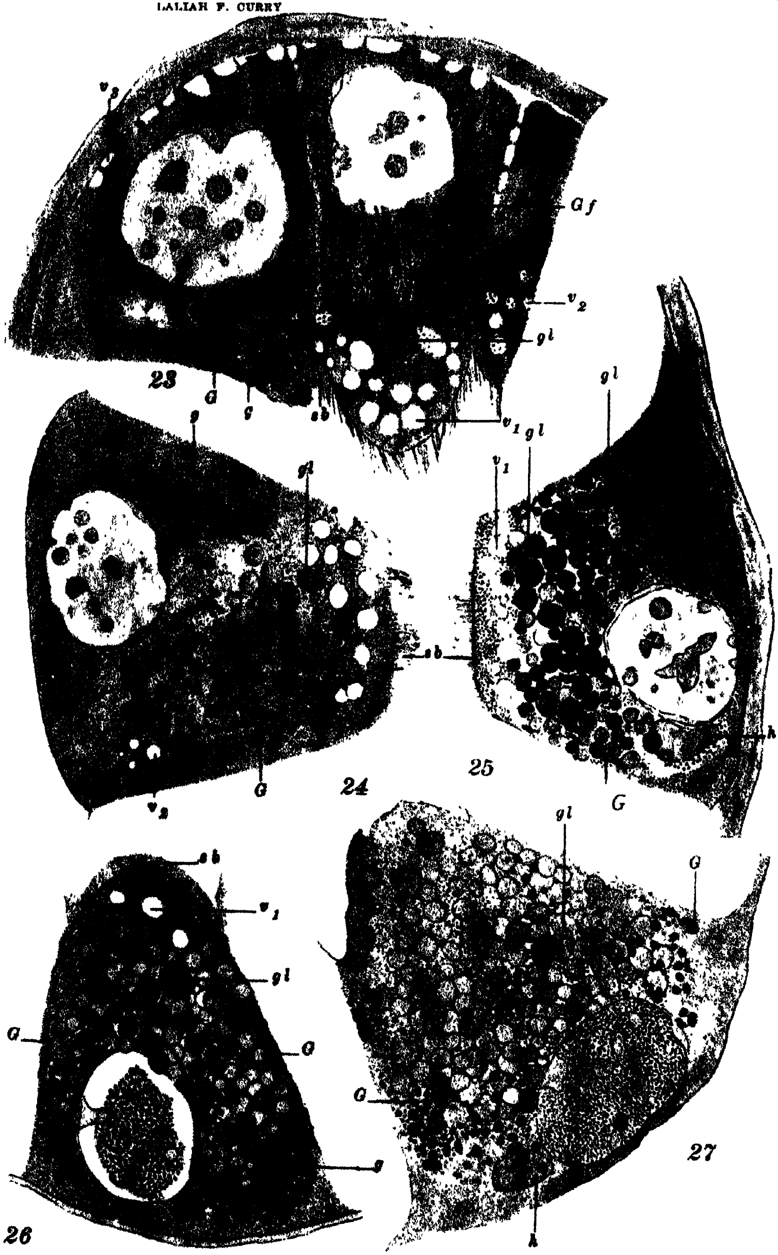
23 Many small granules fill the supranuclear region of the cell; a few large globules can also been seen. Some small granules, the shape of which was difficult to determine, show adhering minute pieces of Golgi substance. Vacuoles of types 2 and 3

24 Medium number of small granules in upper nuclear region; large globules in supranuclear region. Golgi substance not definitely associated with either.

25 No small granules; large globules fill the entire nuclear region. Fragments of Golgi substance scattered in supranuclear region.

26 Many globules; few granules; small amount of Golgi substance. (Necturus no. 66.)

27 Cell from same slide as figure 26, showing a greater number of large globules and no small granules, and a smaller amount of scattered Golgi substance.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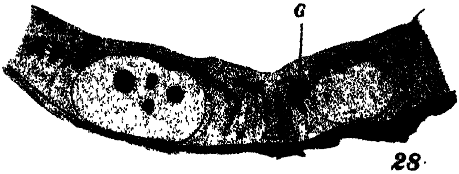

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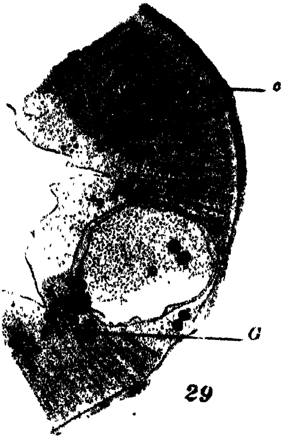

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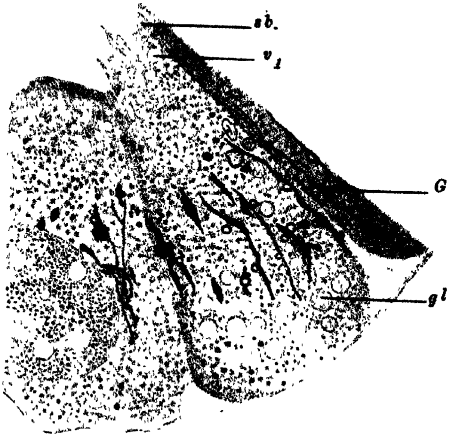

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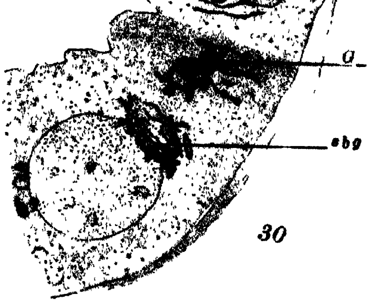

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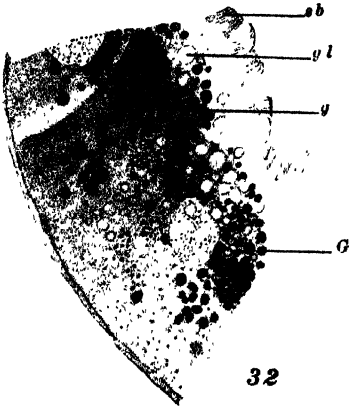

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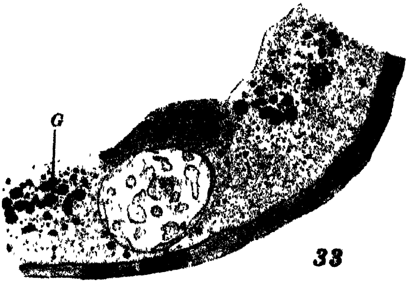

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PLATE 6

EXPLANATION OF FIGURES

These drawings illustrate the diversity of appearance of Golgi substance in different individuals, and the similarity of its appearance in cells of the distal and proximal tubules of the same animal.

28 and 29 Distal-tubule cells of animal no. 66 (see figs. 26 and 27 for proximal tubule cells of the same animal); show similarity of form in cells of very different sizes.

30 Distal-tubule cell of animal no. 42; long filamentous Golgi bodies. Essentially similar in appearance to those found in the proximal-tubule cells of the same animal (fig. 31).

31 Proximal-tubule cell of animal no. 42, showing similarity in form of the Golgi substance to that in the distal-tubule cells of the same animal (fig. 30).

32 and 33 Proximal- and distal-tubule cells of animal no. 61, showing characteristic appearance of the Golgi substance occurring in both types of cells.

PLATE 7

EXPLANATION OF FIGURES

These drawings illustrate a series of reciprocal changes in the number of globules and the amount of Golgi material occurring in cells of the same individual. (Necturus no. 87.)

34 Small proximal-tubule cell with no granules; two large globules, and several large vacuoles which may be spaces left by dissolved globules; one small globule within a vacuole.

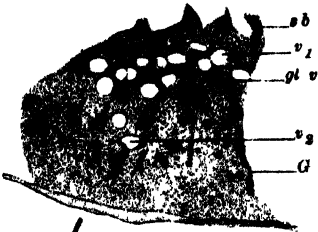
35 Proximal-tubule cell; medium number of granules, few crystals. Nucleus partly hidden.

36 Proximal-tubule cell; numerous granules and crystals; large globules in supranuclear region.

37 Very large proximal-tubule cells; very large globules; some filamentous Golgi bodies.

38 Distal-tubule cell; filamentous Golgi bodies in supranuclear region.

39 Distal-tubule cell; abundant filamentous Golgi bodies in nuclear region.



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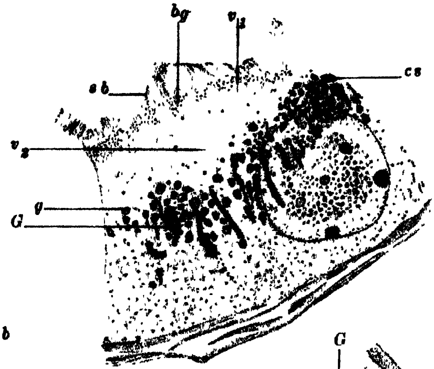

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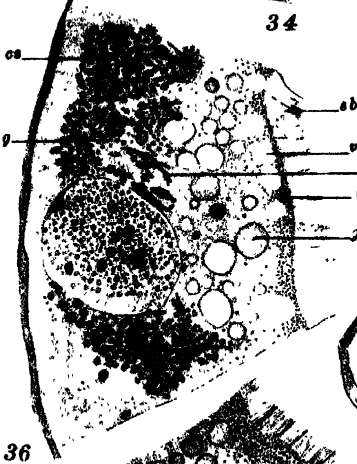

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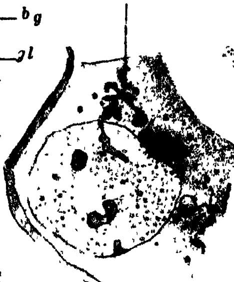

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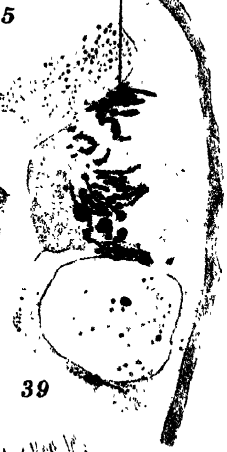

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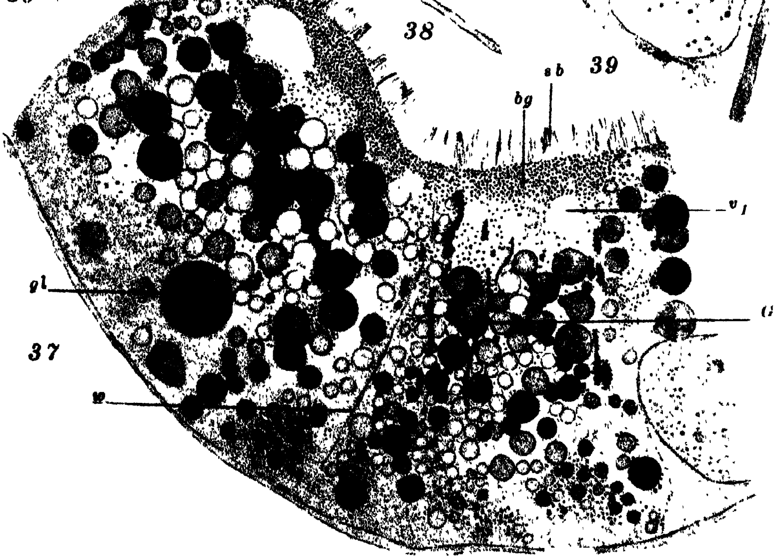

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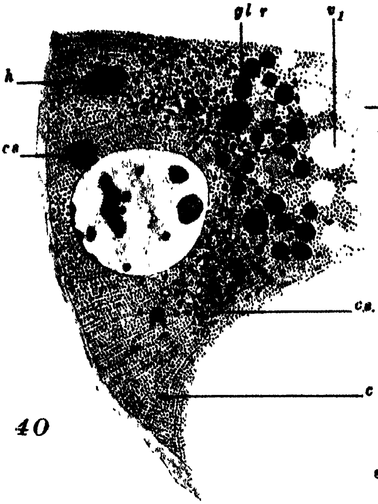

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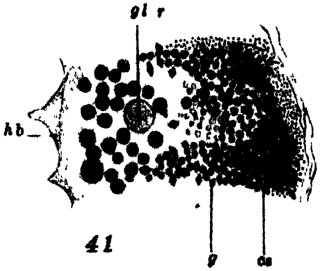

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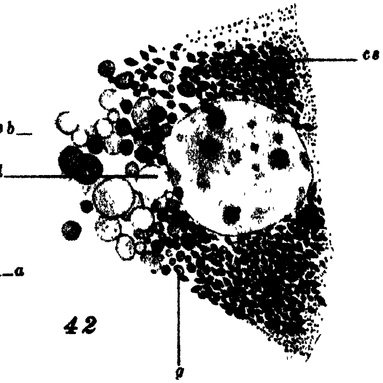

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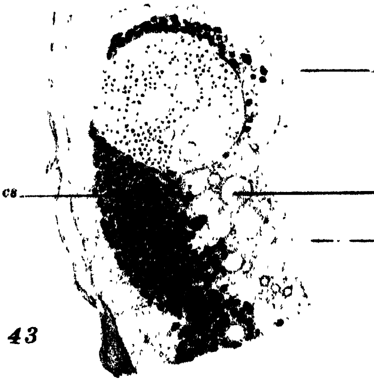

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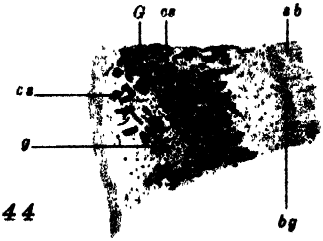

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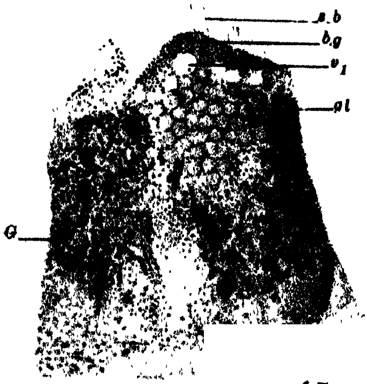

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PLATE 8

EXPLANATION OF FIGURES

All drawings represent proximal-tubule cells.

40 Small green spherical granules, and comparatively few crystals dispersed with them. A group of crystals near the nucleus. (Necturus no. 40; Champy.)

41 Small cell; mixture of spherical granules and crystals closely packed together. (Necturus no. 80; Regaud.)

42 Very many crystals; few small spherical granules and globules. (Necturus no. 60; Regaud.)

43 Many crystals in material fixed by the Mann Kopsch method. Part of the cell is covered by an abnormal cell. (Necturus no. 88.)

Drawings 44 to 46 represent proximal-tubule cells of material fixed by the Mann-Kopsch method from animals injected with large amounts of sugar.

44 Characteristic cell of animal injected with glucose; few large crystals distinguishable. (Necturus no. 139.)

45 This cell shows the characteristic appearance of the osmiophilic substances in Necturus no. 132.

PLATE 9

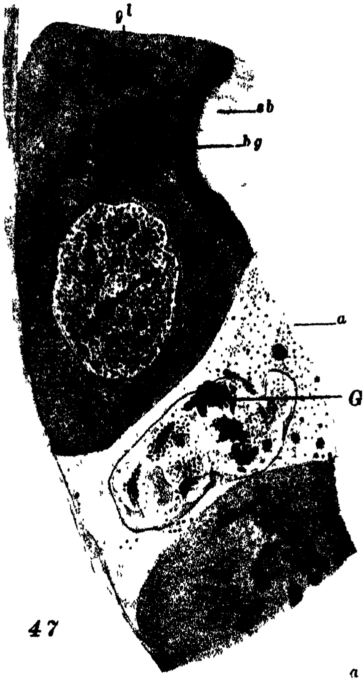
EXPLANATION OF FIGURES

All drawings represent proximal-tubule cells.

46 This cell shows the unusual osmiophilic bodies separated from each other and with a slightly different appearance than in figures 43 and 44. (Necturus no. 134.)

47 Cells of an untreated animal; cell with slightly concave brush border, and, next to it, an abnormal cell with no brush border. The latter is an unusual one of its type in having abundant Golgi substance. (Mann-Kopsch.)

48 Cells from Necturus no. 138 fixed by the Mann-Kopsch method, showing the characteristic appearance of the Gólgi substance of the cells of this animal, contrasting with figure 46. Examples of various relations of abnormal cells without brush border to normal cells.



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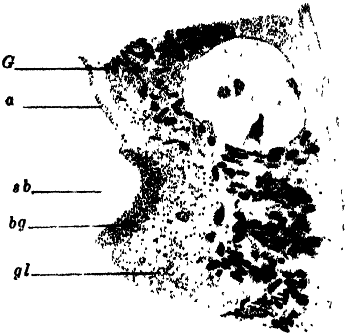

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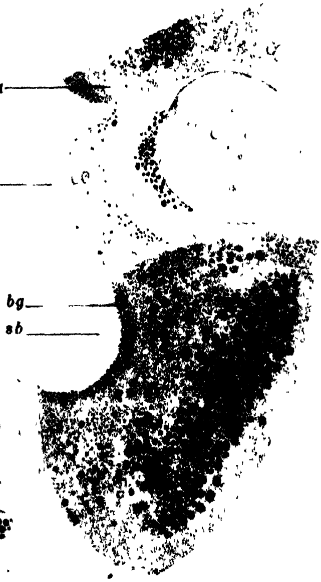

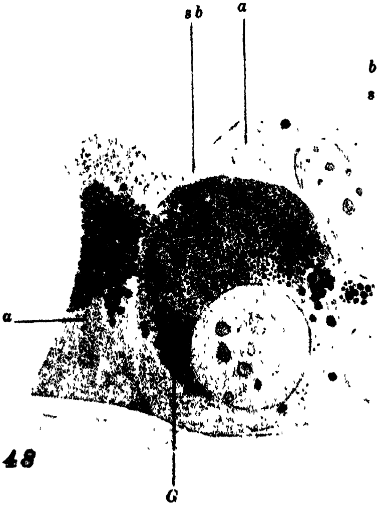

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STUDIES IN THE ORIGIN OF YOLK

IV. OOGENESIS OF PERIPLANETA AMERICANA

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Department of Zoölogy, Government College, University of the Punjab, Lahore, India

TWENTY-FIVE FIGURES

AUTHORS' ABSTRACT

Technique. This work is based not only on the fixed preparations, but also on fresh cover slip preparations treated with neutral red or 2 per cent osmic acid for a short time.

Golgi elements and fatty yolk. The Golgi elements are hollow vesicular bodies with a distinct osmiophilic rim and a central osmiophobic substance. In the youngest oocyte they form a circumnuclear ring. Gradually the vesicles spread out, grow in size, store up fat in their interior, and give rise to the fatty yolk On account of their higher refractive index, due to the presence of fat, the Golgi vesicles can be occasionally seen even in the young oocytes without any treatment.

Mitochondria. The mitochondrial granules also form a circumnuclear ring and are later distributed uniformly.

Albuminous yolk. The albuminous yolk is nucleolar in origin. Early in oogenesis, the nucleolus buds off small, homogeneous, and highly chromatic particles in the cytoplasm, which sooner or later disappear Subsequently, the nucleolus becomes less chromatic and develops vacuolar bodies in its interior, which, becoming vacuolated exactly like the parent nucleolus, migrate into the cytoplasm. These bodies become more and more chromatic and travel toward the periphery of the egg, where they grow in size. Ultimately they break down into small, homogeneous, and highly chromatic bodies which are the definitive albuminous yolk spheres and which subsequently grow enormously in size.

INTRODUCTION

In the heat of the controversy on the shape of the Golgi apparatus in the metazoan cell and its possible homologue in plant and the protozoan cells the most important point must never be lost sight of, namely, that observations based entirely on fixed preparations have little or no value. The elaborate methods of fixation and staining which are now available have demonstrated beyond doubt the existence of the Golgi apparatus in a large variety of cells, but, far from bringing about anything like a general agreement with regard to the shape of the apparatus even in the metazoan cell, they have actually led to an acute difference of opinion on the subject. From this rather disturbing prospect one naturally turns to the study of fresh cells, but here again one finds that in the somatic and the male germ cells the low refractive index of the apparatus does not allow such observations, except in a very few cases for which reference may be made to Bowen ('28). As one of us has pointed out elsewhere (Nath, '28), the egg cells furnish the most favorable material for the study of the Golgi apparatus in the fresh state, because, in addition to their large size, the refractive index of the Golgi elements in such cells is higher on account of the presence of free fat in their interior. This extraordinarily favorable circumstance has enabled one of us (V. N.) to study the Golgi elements in the fresh eggs of spider ('28), in Scolopendra and in Luciola (Quart. Jour. Micr. Sci., in press), and in Culex (Zeit. Zellf. mikr. Anat., Bd. 8, H. 4) with diagrammatic clearness from the youngest to the most advanced oocytes and to show that the Golgi elements are vesicular, with a hollow interior and a definite membrane. In the spider, Scolopendra, and Luciola the Golgi vesicles enlarge considerably when the egg grows, with the result that the membrane of the vesicles becomes more and more attenuated. Pari passu with their growth, free fat not miscible with the general cytoplasm is deposited in the interior of the vesicles. The most remarkable case, however, is that of Culex, in which the Golgi vesicles can be seen with the utmost ease in all stages of the oocyte without the aid either of vital dyes or slight tinging with osmic acid for a short time. If an advanced oocyte of Culex is ruptured on a slide and its contents studied under the microscope, one can observe, in addition to the large albuminous yolk bodies, tiny vesicles performing a dancing movement (due to diffusion currents) which is very interesting to watch. These are the Golgi vesicles, and they react to silver nitrate and osmic acid like the typical Golgi material, each vesicle showing a blackened peripheral rim and a central clear substance. We recommend this material to the most skeptical.

The technique employed for the eggs cited above, as well as for the eggs of the cockroach which forms the subject-matter of the present paper, is simple and can be used even by the beginner. Ovaries are dropped in a very weak solution of neutral red and kept there at room temperature for a short time, the exact period of immersion varying with the material and the strength of neutral red, but generally one hour is quite sufficient. Neutral red is prepared fresh every time it is required, either in distilled water or in normal saline. No definite concentration of the solution is aimed at, but it should be very weak. The Golgi elements are never stained brilliantly by the dye, but they are very slightly and sufficiently tinged to make them visible.

Another technique employed consists of dropping the ovaries in 2 per cent or 1 per cent osmic acid for a short time, the exact period varying with the concentration of the acid and the amount of fat present in the Golgi elements. Ovaries are then studied in a drop of water under a cover-slip. This technique cannot, of course, be called 'vital,' but it comes very near it, for osmic acid used for a short time is the best fixative known, and when it is allowed to act for a short time it does not produce those artifacts which it does in prolonged osmication. The great advantage of this technique is that it enables the worker to distinguish fatty inclusions from those which are not fatty.

In addition to the above technique, we have employed the usual laboratory techniques, namely, the Kolatchev, the Mann-Kopsch, the Champy-Kull, the Da Fano, and the Bouin-iron-haematoxylin.

PREVIOUS WORK ON THE COCKROACH

In a paper on synapsis Hogben ('20) gives a brief account of yolk formation in Periplaneta. The yolk which is albuminous in nature arises from nucleolar extrusions of a remarkable type. At the very beginning of the growth period the highly chromatic nucleolus becomes pear-shaped in outline and emits small, deeply staining, and homogeneous bodies in the cytoplasm which are either ejected from the egg or transformed into a material no longer distinguishable from the ground cytoplasm. At a later stage the hitherto chromatic nucleolus loses its opacity and develops vacuoles inside it. These vacuoles gradually assume a granular and more chromatic form and, boring their way through the nuclear membrane, eventually migrate to the periphery of the egg. This process continues for some time. Ultimately, the nucleolar extrusions break up into several homogeneous globules, which are the yolk spheres sensu stricto. Since Hogben mainly used fixatives containing acetic acid, he has not given any account of the Golgi elements. He has, however, figured minutely granular mitochondria distributed uniformly in a young oocyte, but he has not traced them from the youngest to the advanced oocytes.

We are in substantial accord with the observations of Hogben with regard to the nucleolar origin of albuminous yolk. We have, however, considerably extended these observations and have shown that in the egg of the cockroach there is, in addition to the albuminous yolk, fatty yolk also, which represents the very much enlarged Golgi vesicles containing free fat.

Blochmann ('84, '87), as quoted by Wilson ('25), has described bacterioid forms in the eggs of Periplaneta. He found that the youngest oocytes were free from these bodies, but are later infected with them from the surrounding cells. We have shown that the bacteria always lie at the periphery of the egg, so as to obscure in sections the inner membrane of the follicular epithelium. We have figured them in all but the youngest oocytes, but, since we have paid scant attention to them, we are not in a position to discuss profitably their origin and relationship with the egg.

OBSERVATIONS

Golgi elements and fatty yolk

Fresh cover-slip preparations. It has been possible to study oocytes of all stages in fresh cover-slip preparations of ovaries either stained with neutral red or kept in 2 per cent osmic for some time. Before placing the ovaries in neutral red or osmic acid, it is essential to remove very gently with a brush the haemocoelic packing tissue lying on their surface.

Figure 2 shows the anterior end of an ovarian filament kept in 2 per cent osmic acid for twenty hours, showing ten of the youngest oocytes. The Golgi elements form a dense circumnuclear ring, each appearing as a grayish or slightly copper-colored and refringent vesicle. The fact that the Golgi vesicles do not go black in osmic acid in twenty hours or even in forty-eight hours indicates that there is hardly any free fat inside their interior at this stage. The vesicles are so small at this time that their hollow nature cannot be determined. In the same figure the empty space is occupied by the oogonia, which have not been drawn because they con-

ABBREVIATIONS FOR ALL FIGURES

A.Y., albuminous yolk
A.Y.', nucleolar extrusion breaking down
B., bacteria
E.M., egg membrane
F.E., follicular epithelium
F.E.N., follicular epithelium nucleus
G.V., Golgi vesicle
G.V.', Golgi vesicle containing fat
M., mitochondria
N., nucleolus
Nu., nucleus
N.E., nucleolar extrusion
O.F., ovarian filament
S., space occupied by oogonia
T.T., terminal thread

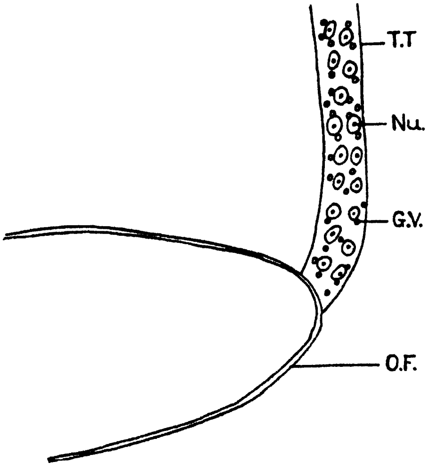


Fig. 1 Kept in 2 per cent osmic acid for forty-eight hours. × 216.

sist of big nuclei and a very small amount of cytoplasm which can hardly be seen in whole mounts. In fixed preparations we have seen the oogonial nuclei in different stages of mitosis (for details of oogonial mitosis refer to Hogben, '20).

We have observed the Golgi elements in the terminal thread by means of which the eight ovarian filaments of an ovary are attached to the wall of the body cavity. A portion of such a thread has been shown in figure 1 (kept in 2 per cent osmic for forty-eight hours). The filament is surrounded by a distinct membrane. Through this thread runs clear protoplasm containing nuclei arranged in an irregular fashion, but one can find no sign of the cell membranes. The

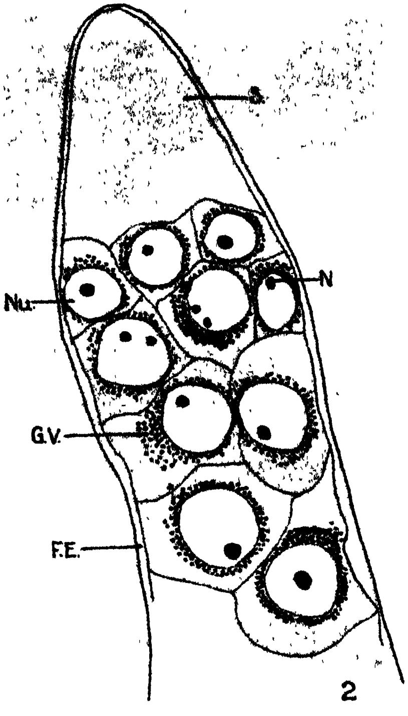


Fig. 2 Kept in 2 per cent osmic acid for twenty hours. × 216.

Golgi elements appear as small, highly refringent, and copper-colored vesicles distributed irregularly throughout the cytoplasm.

With the growth of the oocyte, the circumnuclear ring of Golgi vesicles begins to spread out in the cytoplasm (figs. 3, 4, and 5). All these figures have been drawn from material kept in 2 per cent osmic for forty-eight hours. The Golgi elements appear as slightly blackened vesicles, indicating the existence of small amounts of free fat inside their interior. They have also unmistakably grown in size. It is now possible to study their shape. In the uppermost plane they appear as dark vesicles. When the objective is slightly lowered, they show a dark peripheral ring, due to curvature, and a central less dark area represented in the figures by the white background of the paper. When the objective is still farther lowered, the Golgi elements again appear as dark vesicles, as in the uppermost plane. It must, therefore, be admitted that each vesicle has a distinct osmiophilic membrane and an osmiophobic central substance. If the osmication is prolonged, the vesicles are blackened excessively, or if it is reduced to twenty-four hours or less, they appear copper-colored. In both cases it is difficult to determine their hollow nature. One, therefore, has to find out the period of optimum osmication, which is about forty-eight hours for the stages represented in figures 3, 4, and 5. The hollow vesicular shape of the Golgi

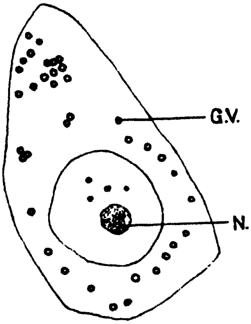
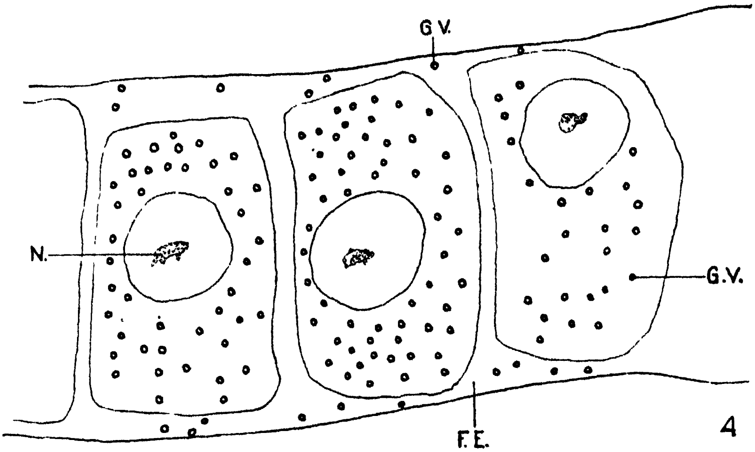


Fig. 3 Kept in 2 per cent osmic acid for forty-eight hours. × 216

elements of the cockroach, as also of those of the spider, of Scolopendra, and of Luciola, reminds one of the mitochondrial vesicles of the male germ cells of scorpions and many insects, each mitochondrial vesicle showing a distinct chromophilic rim and a central chromophobic substance.



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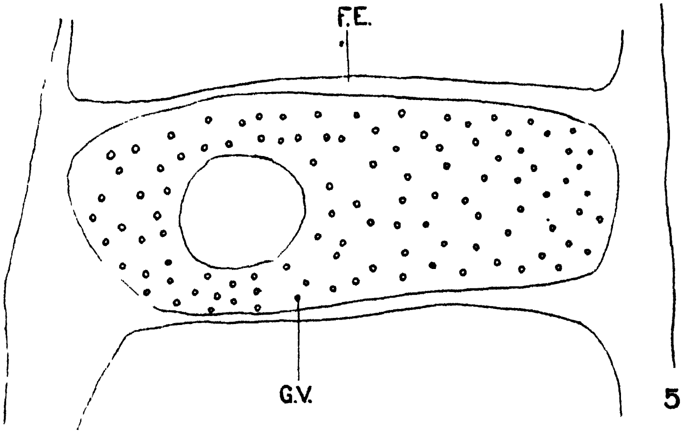

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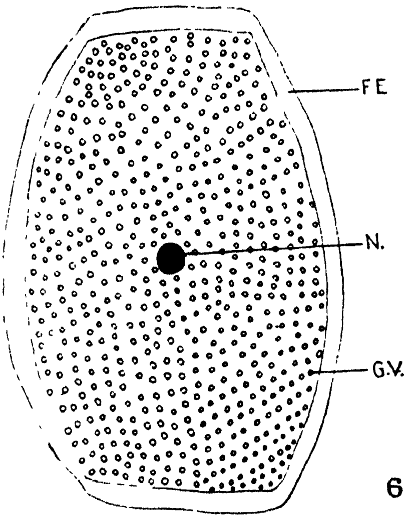
Fig. 4 Kept in 2 per cent osmic acid for forty-eight hours. × 216.
Fig. 5 Kept in 2 per cent osmic acid for forty-eight hours. × 216.

The Golgi vesicles continue to grow in number, and a large number of them grow in size also. At the same time they migrate toward the periphery of the egg and lie below the follicular epithelium. This peripheral arrangement which can be determined in sections (vide infra) continues till the oocyte assumes a length of about 0.7 mm., after which the Golgi vesicles are again uniformly distributed (figs. 6 and 7; kept in 2 per cent osmic for forty-eight hours). By the time the oocyte assumes a length of about 1.5 mm., the albuminous yolk bodies enlarge sufficiently to make the oocyte opaque and quite unfit for study in whole mounts.



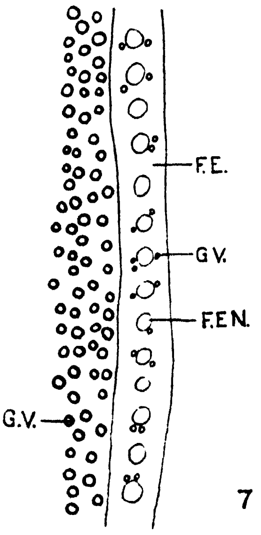


Fig. 6 Kept in 2 per cent osmic acid for forty eight hours. × 56. Length of egg = 1.28 mm.

Fig. 7 Kept in 2 per cent osmic acid for forty-eight hours. × 216.

In figure 7 the follicular epithelium and the periphery of an oocyte (about 1 mm. in length) are shown. The Golgi vesicles of the oocyte are blackened slightly and show the familiar osmiophilic rim and the central osmiophobic substance represented by the white background of the paper.

The Golgi vesicles in the follicular epithelial cells appear copper-colored or, at most, much less dark than those of the oocyte—indicating the presence of much smaller quantities of free fat in their interior. They are also much smaller in size than the vesicles of the oocyte.

It has been mentioned above that when the albuminous yolk bodies enlarge they make the oocyte opaque and unfit for study in whole mounts. We are therefore compelled to rupture the egg, allow its contents to spread out on the slide, and then study them under the microscope. This operation may appear crude, but this has proved eminently satisfactory, as it eliminates the serious artifacts resulting from mechanical displacement caused by the knife in section cutting. In figure 9 are shown the contents of an oocyte about 1.5 mm. and kept in 2 per cent osmic for forty-three hours. The striated spheres represent the albuminous yolk and they appear solid and yellowish in color. Some of the Golgi vesicles (*G.V'.*) have increased in size, and the fact that they have completely gone black unmistakably points toward the existence of free fat in their interior. The rings (*G.V.*) represent those Golgi vesicles which are not very fatty, because they are much less dark. Consequently, they appear, as in younger oocytes, with the familiar osmiophilic rim and a central osmiophobic substance. In figure 10 are represented the contents of the largest ovarian oocyte (4 mm. long) kept in 2 per cent osmic acid for forty-eight hours. The fatty Golgi vesicles (*G.V'.*)

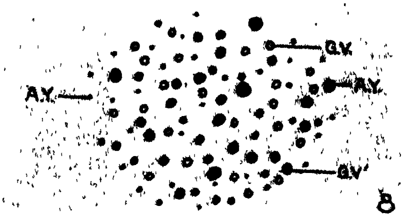
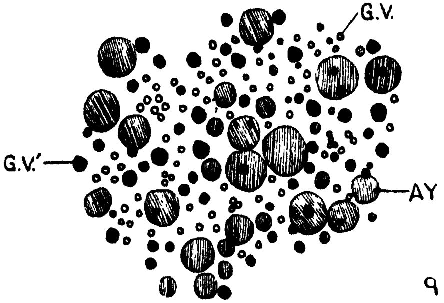


Fig. 8 Kept in 2 per cent osmic acid for twelve hours at 30°C. × 328. Length of the egg = 0.692 mm.

have grown considerably and may now be called the fatty yolk spheres, whereas the non-fatty vesicles (*G.V.*) or vesicles with smaller amounts of fat in their interior become only slightly black, with the result that it is possible to study their hollow nature. Although in this and the previous diagrams the non-fatty vesicles have been shown as rings, it must be



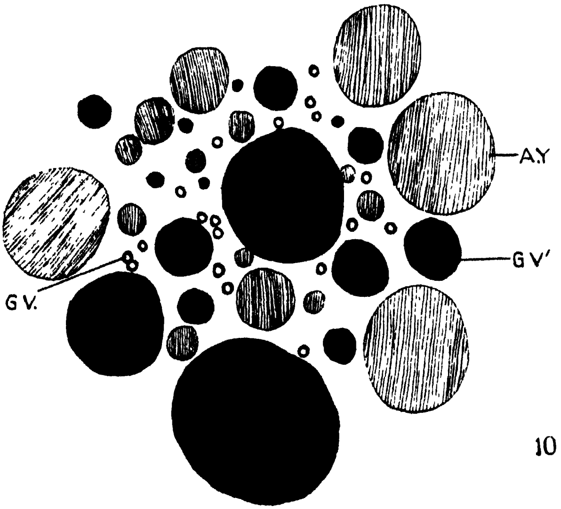


Fig. 9 Kept in 2 per cent osmic acid for forty-three hours. × 216. Length of the egg = 1.5 mm.

Fig. 10 Kept in 2 per cent osmic acid for forty-eight hours. × 216. Length of the egg = 4 mm.

clearly understood that they are really vesicular, the outer rim at the point of curvature being represented as a black line and the central hollow and less dark substance being represented by the white background of the paper. We would have preferred to shade this central area lightly, but we have deliberately refrained from doing so to avoid unnecessary difficulties in the matter of reproduction. Figure 8 represents the contents of a ruptured oocyte (0.692 mm. long) kept in 2 per cent osmic acid for twelve hours at 30°C. Oocytes of this size and even larger ones (fig. 6; 1.28 mm.) certainly show the Golgi vesicles even in whole mounts, but the albuminous yolk bodies which are still very small do not appear, especially because osmic acid does not at all blacken them. The contents of such oocytes may also be spread out on the slide, as in the case of more advanced oocytes, to enable one to see the small yellowish solid albuminous yolk bodies in

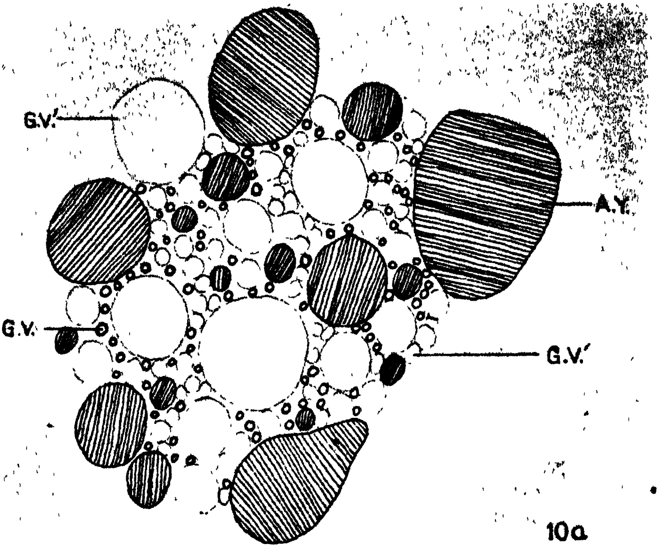


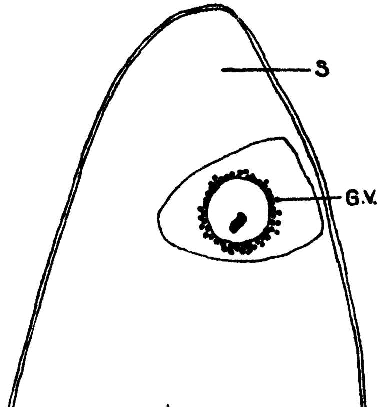
Fig. 10 a Kolatchev. Decolorized in turpentine. × 216.

addition to the Golgi vesicles which are osmicated in different degrees, according to the amount of free fat in them.

It has also been possible to study the Golgi elements in whole mounts with neutral red up to the stage when the oocytes become opaque as the result of the enlargement of the albuminous yolk spheres. We have kept the ovaries in a very weak solution of neutral red for about an hour only. With longer periods the whole oocyte becomes opaque as the result of overstaining of the cytoplasm which seems to mask the Golgi vesicles in spite of their refrangibility. In the period of one hour, however, the dye very slightly but sufficiently tinges the Golgi vesicles, both in the oocyte itself and the follicular epithelium (figs. 11, 12, 13, and 14). Although neutral red is useful in the study of Golgi vesicles, it naturally fails to show whether the vesicles at a particular stage are fatty or non-fatty. On the other hand, osmic acid employed for a short time is a valuable reagent to show the existence of fat in the vesicles by blackening them.

We have several times mounted the proximal portions of the ovarian filaments directly in a drop of distilled water without previously keeping them either in neutral red or osmic acid and have sometimes seen the Golgi elements as refringent, grayish vesicles lying in the cytoplasm, which also appears grayish. We have certainly not succeeded every time we have mounted the ovaries in the manner described, but, provided there is not unduly excessive light falling on the eyes of the observer, success can sometimes be achieved.

Fixed preparations. We have used the Kolatchev, the Mann-Kopsch, the Da Fano, and the Champy-Kull methods for the study of the Golgi elements in fixed preparations. In the first method the best preparations are obtained after four days' incubation. If the period of incubation is prolonged, there is not only a considerable amount of shrinkage both of the cytoplasm and the Golgi elements, but, what is very important to note, the latter become distorted and appear as irregular bodies. In the Mann-Kopsch method, also, the period of osmication must be reduced to about twelve days. We tried about twenty days and discovered a considerable amount of shrinkage of the vesicles, but they are not



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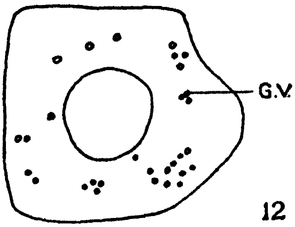

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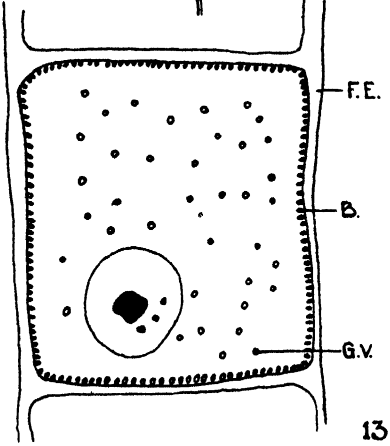

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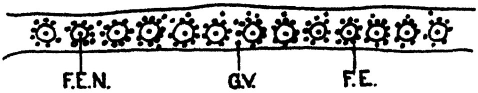

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Fig. 11 Kept in neutral red for one hour. × 216.
Fig. 12 Kept in neutral red for one hour. × 216.
Fig. 13 Kept in neutral red for one hour. × 216.
Fig. 14 Kept in neutral red for one hour. × 216.

distorted as they are after more than four days' incubation in the Kolatchev method. Higher temperature (Kolatchev's method), therefore, not only causes a shrinkage of the vesicles as prolonged osmication in the Mann-Kopsch method does, but it produces distortion also—a much more serious artifact. In the Da Fano method the Golgi vesicles are blackened, but here also there is a considerable amount of shrinkage.

Figures 15, 16, 17, and 18 (Kolatchev) illustrate the primary circumnuclear arrangement of the Golgi vesicles and the mitochondrial granules and their subsequent uniform dispersal in the cytoplasm. In four days' incubation the Golgi vesicles are completely blackened and they, therefore, appear solid. If the osmication, however, is lighter, as in the Champy-Kull preparations (fig. 21), the hollow nature of the Golgi vesicles can be easily ascertained by raising and lowering the objective.

Some time after the stage represented in figure 18, the Golgi vesicles—at least the majority of them—migrate toward the periphery (fig. 22; Champy-Kull). This peripheral arrangement of the Golgi vesicles continues for some time and they are again uniformly distributed about the time the albuminous yolk puts in its appearance (fig. 19; Kolatchev).

From the time the circumnuclear ring of Golgi vesicles breaks away from the nuclear membrane, the majority of the vesicles continue to grow in size and become more and more fatty till in the most advanced oocyte (about 4 mm. long) they assume huge dimensions (fig. 10). Pari passu with this enlargement the membrane of the Golgi vesicle becomes more and more attenuated. These enlarged Golgi vesicles are the fatty yolk. In the nature of the case it is not possible for us to say at what stage of swelling and deposition of fat in its interior a Golgi vesicle should be called fatty yolk. In Kolatchev preparations decolorized in turpentine the fatty yolk spheres (*G.V'.*) appear in advanced oocytes (fig. 10 a) as clear vacuoles of different sizes, giving a frothy appearance to the whole egg. The non-fatty Golgi vesicles (*G.V.*) which, previous to treatment with turpentine, appear black

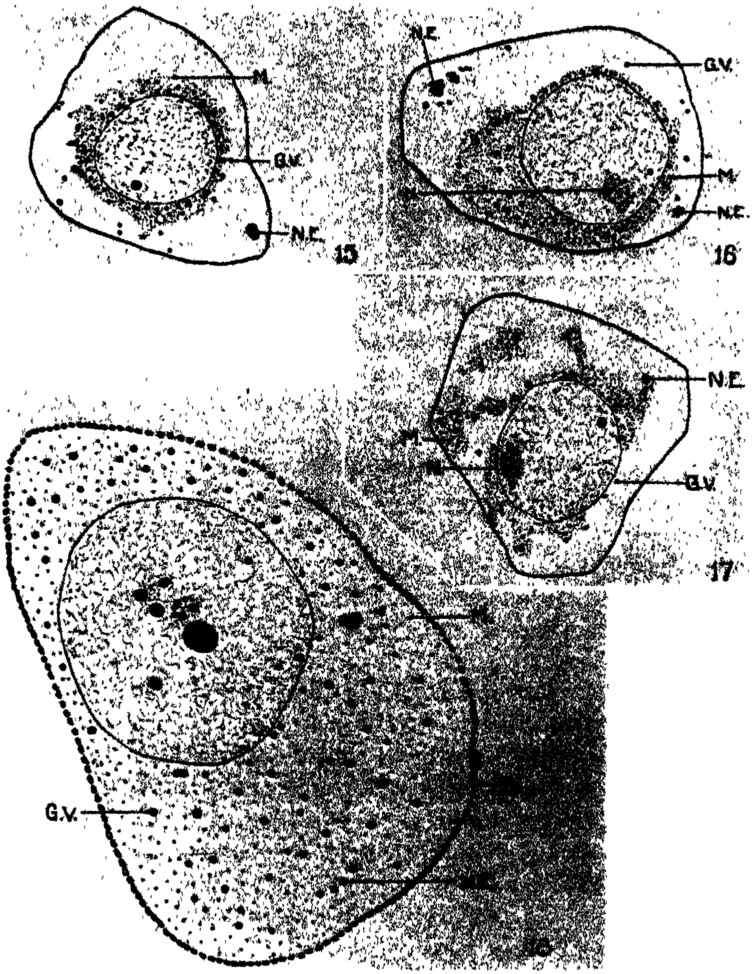


Fig. 15 Kolatchev. Stained with acid fuchsin. × 448.
Fig. 16 Kolatchev. Stained with acid fuchsin. × 448.
Fig. 17 Kolatchev. Stained with acid fuchsin. × 448.
Fig. 18 Kolatchev. Stained with acid fuchsin. × 448.

and solid, appear, after such treatment, distinctly hollow, showing the familiar osmiophilic rim and the central osmiophobic substance. Even in Bouin's preparations the fatty yolk appears in the form of clear vacuoles whose fatty contents have been completely washed out by acetic acid.

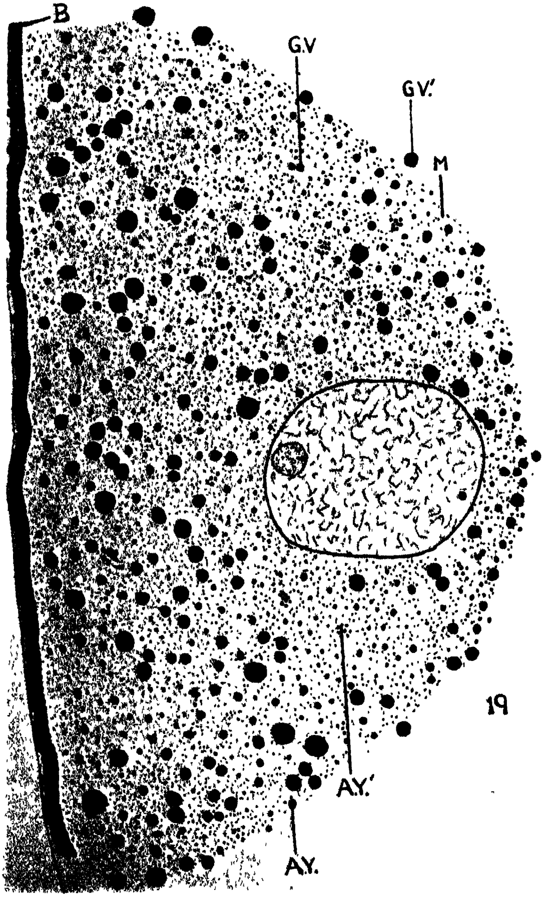
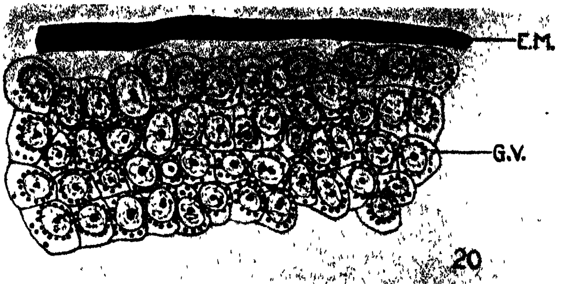


Fig. 19 Kolatchev. Stained with acid fuchsin. × 288. Length of the egg = 0.77 mm. Only a portion of the egg is drawn.

Mitochondria

There is nothing interesting about the mitochondria. In the youngest oocyte they consist of fine granules forming a circumnuclear ring like the Golgi vesicles (figs. 15 and 16). Subsequently, the ring breaks away and the mitochondrial granules are collected in patches (figs. 17 and 21). Ultimately, they are uniformly distributed throughout the cytoplasm as very fine granules (figs. 18, 19, and 22).



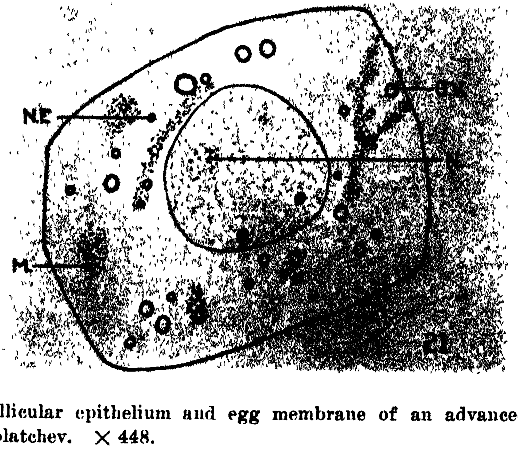


Fig. 20 Follicular epithelium and egg membrane of an advanced oocyte cut obliquely. Kolatchev. × 448.
Fig. 21 Champy-Kull; unstained. × 448.

Nucleolus, nucleolar extrusions, and albuminous yolk

In the youngest oocytes the nucleolus is a spherical structure (fig. 2). Very soon, however, it becomes irregular in outline and, more often than not, it is definitely pyriform. In Bouin-haematoxylin preparations (fig. 23) it appears as a highly chromatic, uniformly staining structure. At the earliest stages of the growth period and before the Golgi-mitochondrial ring has broken away from the nuclear membrane, the nucleolus buds off small, solid, homogeneous, and deeply staining particles into the cytoplasm (figs. 15 to 18 and 23

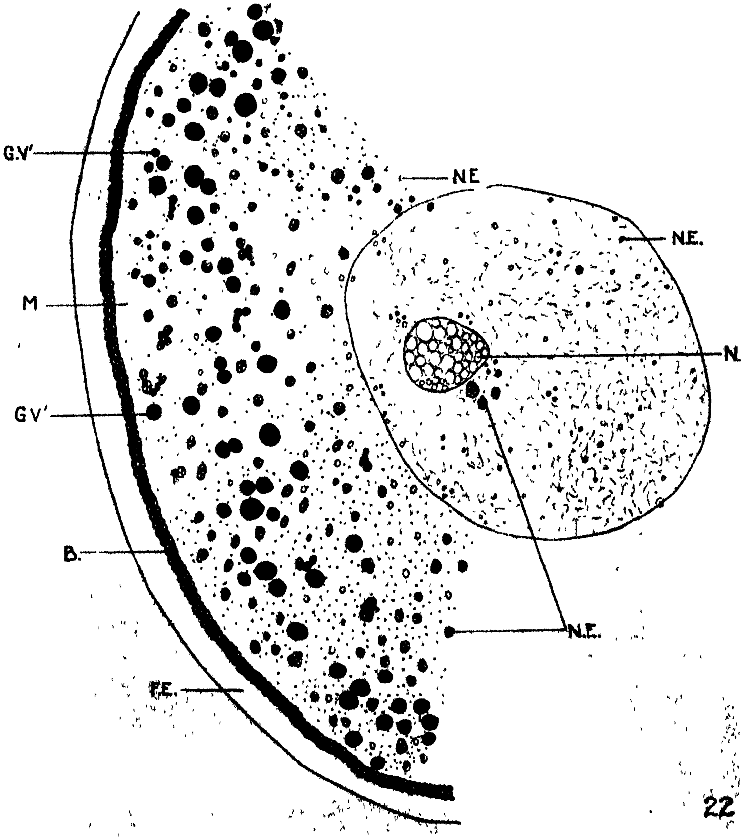


Fig. 22 Champy-Kull; stained with acid fuchsin. × 448. Length of egg = 0.2 mm.

and 24). In the nature of the case it is not an easy matter to determine whether these extrusions proliferate in the cytoplasm or not. But certain appearances seem to support the former view. In figure 16, at *N.E.*, are shown irregular fuchsinophil masses of nucleolar substance. We are inclined to interpret such appearances as the breaking down of an extrusion into smaller ones. Nevertheless, the possibility of small extrusions running together in an artificial manner cannot be entirely excluded.

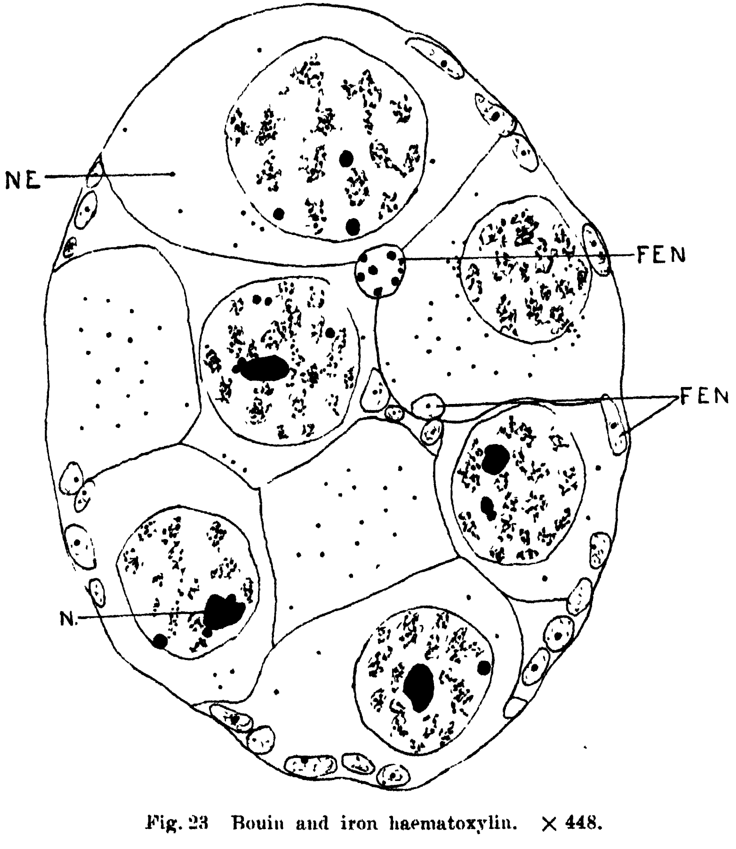


Fig. 23 Bouin and iron haematoxylin. × 448.

Sooner or later, however, these extrusions entirely disappear from the cytoplasm. Before they disappear completely, certain vacuoles appear at the periphery of the egg (fig. 24).

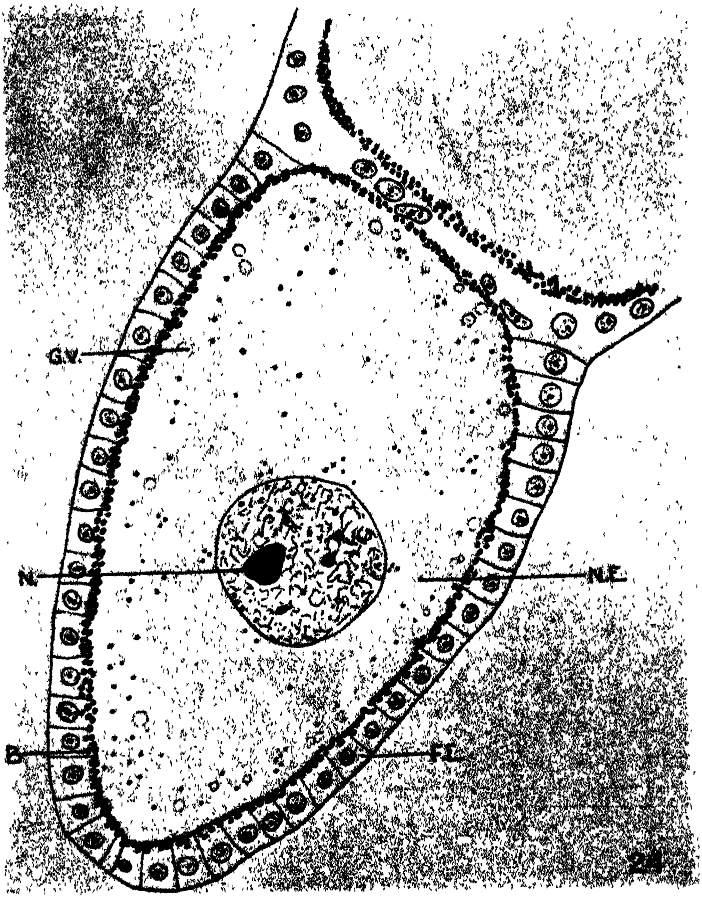


Fig. 24 Bouin and iron haematoxylin. × 288. Length of the egg = 0.184 mm.

Hogben interprets these as the cytoplasmic vacuoles, but they are in reality the Golgi vesicles that have escaped complete washing out in acetic acid. As has been shown in an early section, the Golgi vesicles migrate to the periphery of the egg after their uniform dispersal, where they remain till the egg has assumed a length of about 0.77 mm. Experience shows that acetic acid may not completely wash out closely aggregated Golgi vesicles or mitochondria. From now onward, the nucleolus which was hitherto irregular in outline becomes perfectly spherical. At the same time, it no longer stains uniformly, but shows vacuolar bodies in it. These vacuolar bodies migrate into the cytoplasm either directly or after they have become vacuolated exactly like the parent nucleolus itself (fig. 22). Ultimately, however, all the nucleolar extrusions become vacuolated like the parent nucleolus. As is the case with the Golgi vesicles, these extrusions travel toward the periphery of the egg and lie just below the follicular epithelium (fig. 25). This peripheral migration seems probably to be due to the fact that nutritive fluid flows to the egg from the outside through the follicular epithelial cells, and the periphery of the egg is therefore the most favorable place where the nucleolar extrusions and the Golgi vesicles can grow.

The vacuolated nucleolar extrusions become more and more chromatic and stain deeply with Bouin-haematoxylin, so much so that when they take up the peripheral position they appear as homogeneous bodies, although in reality they are not so. The high chromaticity and the consequent apparent homogeneity of the extrusions may easily mislead one to regard them as the definitive albuminous yolk bodies. But, if the stain is extracted from them, they appear as vacuolated bodies exactly like the parent nucleolus (fig. 25). In this egg (0.4 mm. long) the nucleolus has practically ceased throwing out the characteristic extrusions and the peripheral migration of the latter has been almost completed.

According to Hogben, these extrusions "break up into several homogeneous globules, which are the yolk spheres *sensu stricto.*" Unfortunately, the biggest oocyte which Hogben has figured corresponds roughly to our figure 22, when the egg has become 0.2 mm. long. Nor has he given any magnifications for the figures of the comparatively young oocytes that he has actually drawn. Consequently, it is difficult to understand from his account in what precise manner the homogeneous albuminous yolk bodies arise from the vacuo-

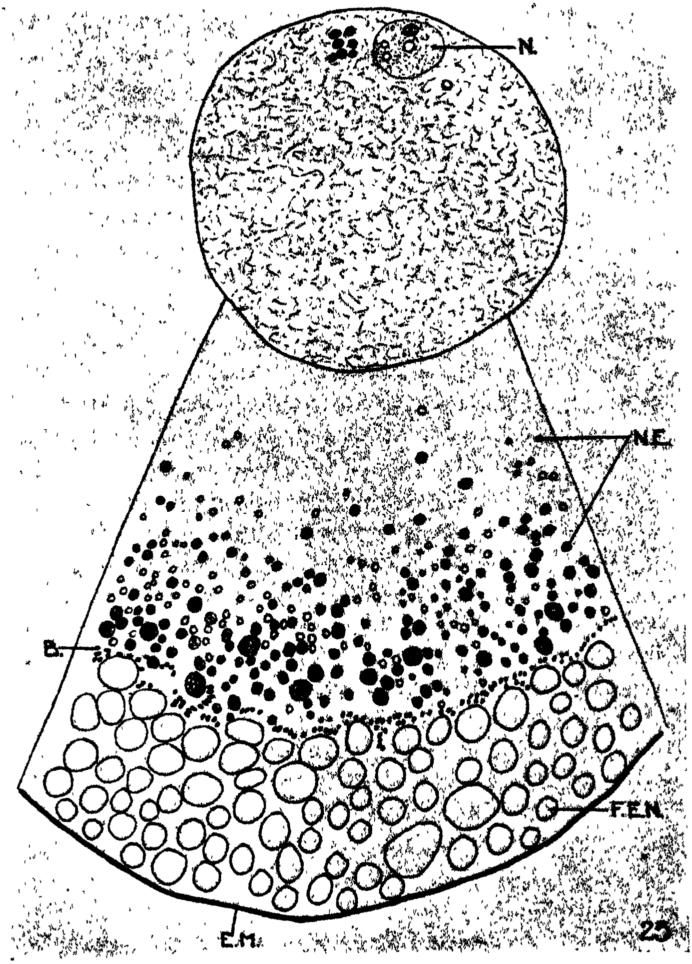


Fig. 25 Bouin and iron-haematoxylin. Understained. × 448. Length of the egg = 0.4 mm.

lated extrusions. As Hogben has correctly pointed out, there is certainly no constant relationship between the size of the egg and the first appearance of albuminous yolk. Nevertheless, measurements of the eggs have considerably helped us to follow the behavior of the nucleolar extrusions.

In figure 22 the egg is about 0.2 mm. long and the vacuolar and the vacuolated nucleolar extrusions are distributed uniformly throughout the cytoplasm. Sooner or later, this stage is followed by one represented in figure 25 (length of egg, 0.4 mm.), when the extrusions are arranged at the periphery of the egg, where they unmistakably grow in size (compare figs. 22 and 25). After they have completed their growth, the extrusions are once more uniformly distributed. We have not figured this stage, because it is exactly like the one represented in figure 22, with the difference that the extrusions are considerably bigger and the nucleolus has ceased budding. When the egg becomes about 0.77 mm. long (fig. 19), the nucleolar extrusions break down into small perfectly homogeneous granules which represent the definitive albuminous yolk. That the extrusions first break down and then give rise to yolk, and are not directly metamorphosed into it, is proved by the fact that the earliest yolk granules (fig. 19) are much smaller than the nucleolar extrusions (fig. 25) and that in figure 19, which is an excellently stained Kolatchev preparation in our possession, the small yolk granules are collected together at several places (*A.Y'.*) probably representing extrusions in the act of breaking down.

According to Hogben, the nucleolar vacuoles gradually assume a granular and more chromatic form, and ultimately break down into several homogeneous globules which are, strictly speaking, the yolk granules. According to our observations, however, the nucleolar vacuoles become themselves vacuolated exactly like the parent nucleolus either before their migration into the cytoplasm or after it. The very small vacuoles in the nucleolar extrusions gradually become more and more chromatic and homogeneous till they give rise, at the time of the breaking down of the extrusions, to yolk globules. The difference in the two accounts is, however, more apparent than real, because Hogben has actually figured (refer to his fig. 31) vacuolated nucleolar extrusions, the small vacuoles inside which will appear like granules when the extrusion becomes highly chromatic.

Although cytologists have long believed that the nucleus plays an important part in controlling the nutrition of the egg, Gatenby ('22), working on Saccocirrus, was the first person to show clearly the exact relationship between the nucleus and the process of vitellogenesis. In this archi-annelid Gatenby showed that albuminous yolk originated directly from nucleolar extrusions. Nath ('24) and King ('24) described simultaneously a similar process in Lithobius forficatus. Later, Nath ('25) described an actively budding nucleolus in the eggs of Euscorpins napoli and Buthus judaicus throwing out extrusions in the cytoplasm before the appearance of albuminous yolk and an inactive nucleolus in those genera of scorpions which entirely lacked this type of yolk, namely, Palamnaeus fulvipes madraspatensis and P. swammerdami. More recently, Nath and Mehta ('27; also Quart. Jour. Micr. Sci., in press) have described albuminous yolk originating from nucleolar extrusions in the eggs of the firefly, Luciola gorhami.

The most remarkable feature of the nucleolar extrusions of the second series in the cockroach, however, is that they are definitely vacuolar bodies developing at a later stage small vacuoles inside them exactly like the parent nucleolus and ultimately becoming homogeneous at the time of their breaking down into the definitive yolk globules.

SUMMARY

1. The oogenesis of the cockroach has been worked out by fresh cover-slip preparations as well as by the usual methods of fixation and staining.

2. On account of their higher refractive index due to the presence of fat, the Golgi elements can be occasionally seen in the young oocytes without the aid of any vital dye.

3. Neutral red tinges the Golgi elements slightly but sufficiently.

4. Immersion of the ovaries in 2 per cent osmic acid for a short period slightly blackens the Golgi elements.

5. The Golgi elements are hollow vesicular bodies with a distinct osmiophilic rim and a central osmiophobic substance.

6. In the youngest oocyte the Golgi vesicles form a circumnuclear ring. Gradually, the ring breaks away from the nuclear membrane and the vesicles are distributed throughout the cytoplasm. With the growth of the oocyte, a large number of Golgi vesicles enormously grow in size, store up fat in their interior, and give rise to the fatty yolk. In the process of enlargement the rim of the vesicle becomes more and more attenuated.

7. The mitochondrial granules, like the Golgi vesicles, form a circumnuclear ring. Subsequently, they are distributed uniformly in the cytoplasm.

8. Albuminous yolk which has a nucleolar origin is also present in the egg of the cockroach.

9. At the very inception of the growth period the hitherto spherical and highly chromatic nucleolus becomes irregular in outline. It buds off small, homogeneous, and intensely staining particles in the cytoplasm which, sooner or later, disappear.

10. Subsequently, the highly chromatic nucleolus becomes less and less chromatic and vacuolar bodies appear in it. These vacuolar bodies again become vacuolated exactly like the parent nucleolus and travel into the cytoplasm.

11. The vacuolated nucleolar extrusions become more and more chromatic and migrate toward the periphery of the egg, where they grow in size.

12. After their growth is completed, they are again uniformly distributed in the cytoplasm, where they break down into small, homogeneous, and highly chromatic bodies which are the definitive albuminous yolk bodies and which subsequently grow enormously in size.

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ON THE ARTERIAL SYSTEM OF THE LIZARD UROMASTIX HARDWICKII GRAY

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NINE FIGURES

AUTHOR'S ABSTRACT

The arterial system of Uromastix hardwickii retains many primitive features and shows great resemblance and relationship to that of Sphenodon. The heart is typically reptilian. Two systemics and a pulmonary arch arise from the ventricle. The conus arteriosus is absent. The carotids arise together from the right systemic by means of a small common carotis primaria. A very well developed ductus caroticus connects each carotid with the systemic arch of its side The ductus arteriosus (ductus Botalli) is absent.

The dorsal aorta is formed by the union of both the systemic arches. The left systemic joins entire, while the right one, which is termed systemicocarotid trunk, gives off the carotids, the vertebral, a single subclavian, and two pairs of parietal arteries, before the union. Anterior epigastric arteries are altogether absent The dorsal aorta gives off fifteen pairs of parietal arteries which are segmentally arranged All the main branches supplying the alimentary canal and other visceral organs arise independently of one another, there being no such combination as is found in Varanus

The justification for writing this paper has arisen from the fact that Uromastix hardwickii is one of the fairly common lizards of northern India and is a very suitable type for dissection. No detailed description of its blood-vascular system seems to exist. Accounts of certain other lizards are found, which show certain resemblances to this animal, but these descriptions do not completely apply to the present species. It seems desirable, therefore, to record the results of my investigation. I have endeavored to give a fairly detailed and adequate description, with the expectation that it will prove helpful to students working on this animal.

The animals used in this study were collected in the vicinity of Lucknow. I have dissected about three dozen fresh specimens, and, besides ordinary dissections, injections were made through the chief vessels. After the animal is chloroformed, the heart beat continues for a considerable time and the injection mass can run through the whole system with great facility.

Permanent injections were also made which proved very successful, particularly in the arterial system. Minute vessels and the structure of the heart were studied both in gross dissections and in the serial sections. The material for sectioning was chiefly fixed in Bouin's fluid or corrosive sublimate and stained with Delafield's haematoxylin. Most of this material was cut rather thick, usually about 10 μ.

I wish to express my thanks to Dr. G. S. Thapar for his suggestions and for his kindness in loaning me books from his private library to facilitate my work. I take pleasure in acknowledging my indebtedness to Dr. K. N. Bahl for the final revision of a portion of the manuscript.

THE HEART

The external appearance of the heart of Uromastix is typically reptilian. It is about 20 mm. long in a specimen of average size, and the auricles measure 15 mm. across when thoroughly extended. The heart lies considerably forward in the median plane between the lungs, and consists of four chambers, a sinus venosus, two auricles, and a ventricle.

The sinus venosus (fig. 1), which is the largest chamber of the heart, is a prominent thin-walled sac situated on the dorsal side of the heart, lying transversely above the auricles, to which it is closely attached. It is formed chiefly by the confluence of the anterior and postcaval veins and is marked out externally into a large right and a small left portion by a slight constriction in the middle. A transverse slit, the sinu-auricular aperture (fig. 2), situated at the constriction, connects it with the right auricle. This aperture is not guarded by any valve, but its lips are thick and muscular and are thus kept closed, except when the blood is to be forced through it into the right auricle.

The venous blood from the different parts of the body is brought into it by three chief vessels, the right anterior vena cava (fig. 1) and the left anterior vena cava from the anterior regions, and a posterior vena cava from the posterior end of the body.

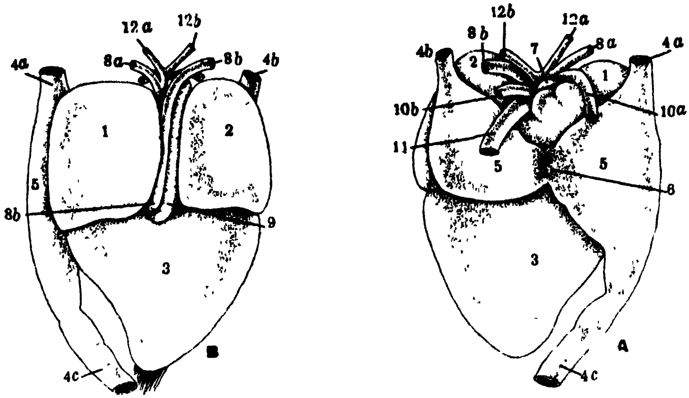


Fig. 1 A. Dorsal view of the heart. B Ventral view of the heart

1, right auricle
2, left auricle
3, ventricle
4a, right anterior vena cava
4b, left anterior vena cava
4c, posterior vena cava
5, sinus venosus
6, constriction
7, diverticulum
8a, right systemic arch
8b, left systemic arch
9, pulmonary arch
10a, right pulmonary artery
10b, left pulmonary artery
11, pulmonary vein
12a, right carotid
12b, left carotid

The right auricle (figs. 1 and 2)

The right auricle is larger than the left. Its walls are thin, with thickenings in several places due to the presence of the interlacing muscular ridges. O'Donoghue(18) describes a sac-like diverticulum in Sphenodon thus:

> From the anterodorsal edge of the auricle, near its middle line is given off a sac-like diverticulum with thin walls. It is seen near the diverging bases of the carotid arteries. It would appear, therefore, as if it was a normal structure, but its function is not obvious.

This diverticulum (fig. 1, *7*), which is a prominent feature in Uromastix, appears to be a simple extension of the auricle. I do not think that it could serve any special function in that place.

The interauricular septum (fig. 2)

The interauricular septum is a thin, muscular partition separating the two auricles. It is situated a little toward the left of the median plane of the heart and is comparatively thinner than the walls of the auricles. This septum ends below in a free posterior edge and divides the auriculoventricular apertures into right and left halves.

The left auricle (figs. 1 and 2)

The left auricle is similar to the right in general structure and appearance, but is slightly smaller. It partly covers the anterior portion of the ventricle. The pulmonary vein opens into it by a circular pulmonary aperture (fig. 2 A, *7*). This is guarded by a valve formed by a forward extension of the dorsal wall of the auricle appearing as a lip-like outgrowth guarding the pulmonary aperture.

The ventricle (figs. 1 and 2)

The ventricle is a thick-walled conical sac with the apex directed backward. It is about 10 mm. long. The color and the thickness of its walls sharply distinguish it from the auricles. The apex of the ventricle bears a small outgrowth connecting it with the pericardium. Beddard(2) describes it as of universal occurrence in Lacertilia and calls it gubernaculum cordis. The walls of the ventricle are spongy with numerous muscle processes, forming an interlacing system of muscular trabeculae. Into the meshes of spongework and interstices the blood can enter for a considerable distance. Both the auricles communicate with the ventricle, and their apertures are separated from each other by a backward extension of the interauricular septum into the ventricle. This septum, as would appear from an ordinary dissection of the heart, bears on either side transverse membranous folds (fig. 2 B), which act as valves guarding the oval openings. These valves are formed by the infolding of the membrane strengthened by muscles and fibrous tissue. The edges of the folds are furnished with a number of delicate threads, the chordae tendineae (fig. 2), which connect them to the walls of the ventricle. The structure thus formed functions as a pocket valve and is interposed in the auriculoventricular apertures. From their structure and position it will be seen that they can allow the flow of blood in one direction only, i.e., from

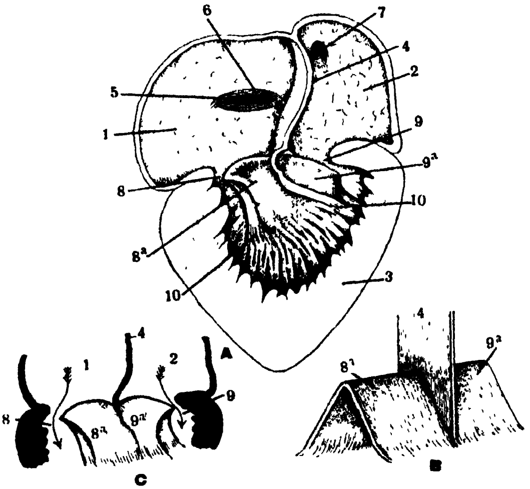


Fig. 2 A. Inner structure of the heart. B. Diagrammatic representation of the auriculoventricular valve. C. Position of the valves.

1, right auricle
2, left auricle
3, ventricle
4, interauricular septum
5, sinu-auricular orifice
6, sinu-auricular muscles
7, pulmonary vein orifice
8, right auriculoventricular orifice
8a, right auriculoventricular valve
9, left auriculoventricular orifice
9a, left auriculoventricular valve
10, chordae tendineae

the auricles to the ventricle, but the backward flow is prevented by the pockets becoming filled with blood and thus blocking the passage (fig. 2 C).

There is no complete partition to divide the ventricle into right and left halves, but an incomplete septum is produced by the attachment of the tendinous cords of the valves of the ventricle, which helps to keep the blood from the two auricles partially separate.

Goodrich(11) and Greil(12) mention similar flaps in Reptilia, and I confirm their statements.

O'Donoghue makes no definite statement, since the state of preservation of the inside of the heart he was working with was not good enough to settle this point satisfactorily.

THE ARTERIAL ARCHES (figs. 1 and 3)

There are three arterial or aortic arches arising independently from the right side of the ventricle: the pulmonary arch; the left systemic arch; the right systemic arch.

The pulmonary and left systemic arches are both visible in a ventral view of the heart, the point of origin of the pulmonary being more toward the right of the left systemic. The right systemic arch lies above the left systemic and the pulmonary artery, and its place of origin, therefore, is not visible from the ventral side (fig. 3 A). Both the right and left systemic arches arise dorsal to the pulmonary arch and then twist round so as to become ventral to it. There is a crossing over of these arches at the distal end when these arches diverge, forming the right and the left systemics (fig. 3 C). Thus it would appear that the right systemic actually arises from the left side and the left systemic from the right side of the ventricle.

While describing the origin of these arches in Sphenodon, O'Donoghue states that—

> The arterial trunks come off from a slight projection of the ventricle which is probably to be regarded as a remnant of the conus arteriosus.

and adds further—

> At the base the three vessels stand in open communication with one another and pass backwards a short distance before their common lumen opens into that of the ventricle and behind this again lies the atrio-ventricular aperture.

He supports this view by mentioning the presence of a pair of semilunar valves at the base. It is quite obvious, therefore, that this portion between the origin of the trunks and the valves cannot be considered as a part of the ventricle. O'Donoghue(18) has given several sketches representing these trunks, which indicate that the *three trunks stand in open communication with one another.*

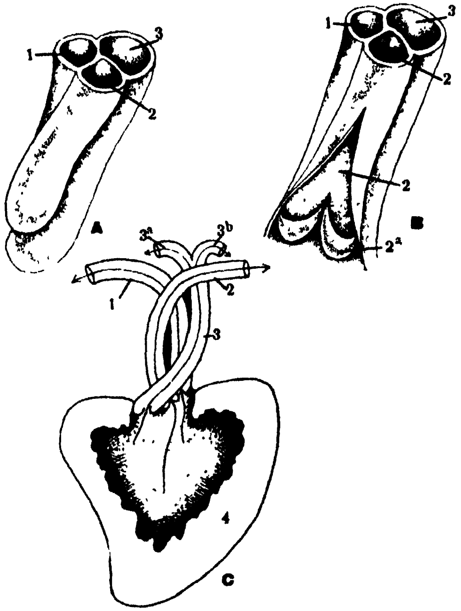


Fig. 3 A. Aortic arches. B. Aortic arches. Left cut longitudinally to expose a pair of valves. C. Diagrammatic arrangement of the aortic arches with their relation to the ventricle, and flow of blood from the ventricle.

1, right systemic arch	*3*, pulmonary arch
2, left systemic arch	*3a*, right pulmonary artery
2a, semilunar valve in the left systemic arch	*3b*, left pulmonary artery
	4, ventricle

As will be seen from the above sketches, the lumens of right and left systemics come together first and the pulmonary joins to the left last of all. Right systemic alone opens into the ventricle and the other two, i.e., left systemic and pulmonary artery, run through it. This led me to study in detail the conditions in Uromastix. By careful dissections and

Fig. 4 Transverse sections through the main arterial trunks, showing their relative position and the position of the valves and their connection with the ventricle.

A. Slide no. 4. Row no. 2. Section no. 7. Section passing through the arches at a level where they are completely separate. Portions of the valves are visible in the right systemic.

1, right systemic arch
1a, right systemic valve
2, left systemic arch
3, pulmonary arch

B. Slide no. 5. Row no. 3. Section no. 5. Section passing through the arch at a level where the pulmonary and the left systemic are independent. Valves are nearly cut away in the right systemic which is in communication with the ventricle. Valves appear in the left systemic as well.

1, right systemic arch
1a, right systemic valve
2, left systemic arch
2a, left systemic valve
3, pulmonary arch
4, ventricle

C. Slide no. 5. Row no. 4. Section no. 2. Section passing through the arches at a level where a septum completely separates the pulmonary artery, while the two systemics are in communication. The valve in the pulmonary arch is also visible.

1, right systemic arch
1a, right systemic valve
2, left systemic
2a, left systemic valve
3, pulmonary
3a, pulmonary valve
4, ventricle

D. Slide no. 7. Row no. 1. Section no. 7. Section passing through the arches at a level where right systemic is no more visible, it is merged into the ventricle; two valves are present still to indicate its position. Left arch is in much greater communication with the right systemic and through it the ventricle. The valves in the pulmonary arch are more developed, and it is still an independent trunk.

1, right systemic arch
2, left systemic arch
2a, left systemic valve
3, pulmonary arch
3a, pulmonary valve
4, ventricle

E. Slide no. 8. Row no. 3. Section no. 4. Section passing through the arches at a level where all the three trunks communicate. The two systemics no more exist, as both of them become gradually merged into the ventricle. The pulmonary trunk, which was an independent trunk so far, communicates with the systemics at this point.

2, left systemic arch
3, pulmonary arch
4, ventricle

F. Slide no. 10. Row no. 1. Section no. 2. Section passing at a level where there is no trace left of the two systemics. Pulmonary is the only trunk in communication with the ventricle. The pulmonary valves are visible no more. Only one pocket valve is left at the base.

3, pulmonary arch
3a, pulmonary valve
4, ventricle

serial sections (fig. 4), I have been able to find out that the lumen of the trunks in Uromastix is not completely separate at the base, but it presents several structural differences from that of Sphenodon.

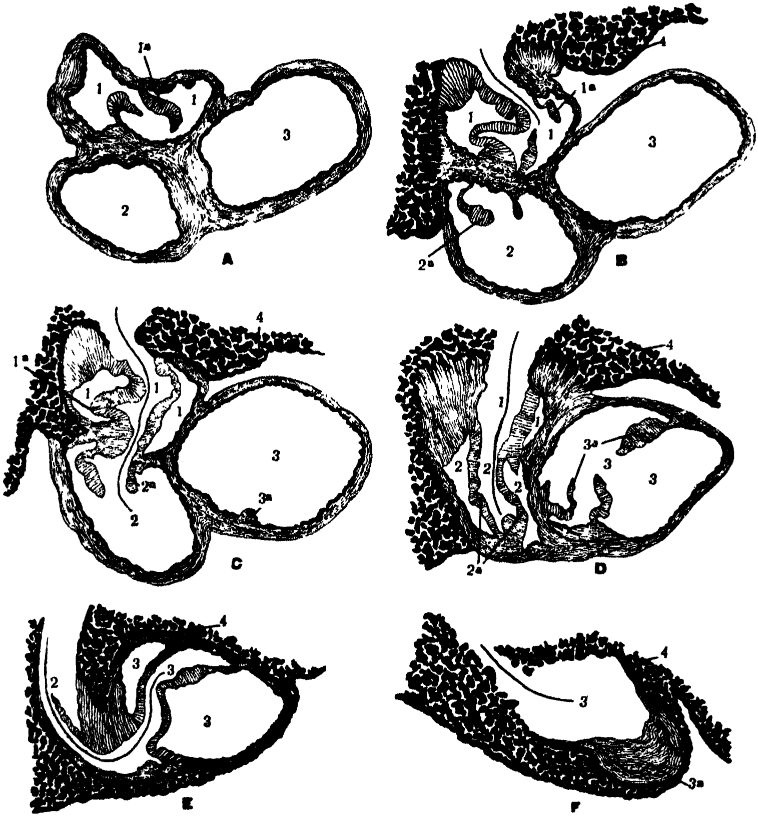


Figure 4

In Uromastix it is the right systemic which opens first into the ventricle. At that place the left systemic and the pulmonary are quite independent. The lumen of the left systemic then becomes connected to the right systemic and, through the latter, to the ventricle, and lastly, the pulmonary also joins to it. The order in which the three trunks run and communicate with each other near the base is, therefore, not the same as in Sphenodon, the difference being that the *lumens of all the trunks in Sphenodon become connected with one another first and then to the ventricle through the right systemic only* (fig. 5 A), *while in Uromastix the right systemic opens first into the ventricle, the left systemic and pulmonary joining later* (fig. 5 B).

This communication of the right arch with the ventricle at a place where the remaining two arches are independent indicates that all the three arches do not stand in direct communication with one another in the same manner described in Sphenodon. Also there are no valves between the base of the arches and the ventricle, and hence no conus arteriosus.

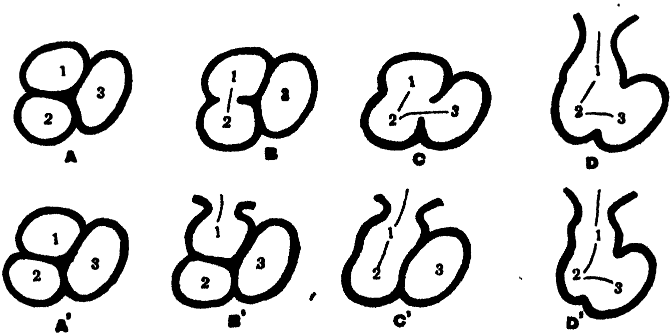


Fig. 5 Diagrammatic representation of the arrangement of the aortic arches in Sphenodon and Uromastix. A, B, C, D, condition in Sphenodon (by O'Donoghue). A', B', C', D', condition in Uromastix.

1, right systemic
2, left systemic
3, pulmonary arch

It will thus be seen that each of the three trunks is independently provided with a pair of semilunar valves, which are formed from the lining membrane, strengthened by fibrous tissue. They are attached by their convex margins to the wall of the vessel, and their free borders are directed forward into the lumen (fig. 3 B). The free and attached margins are strengthened by tendinous fibers. These valves allow the flow of blood from the ventricle to the arches only. It will also appear from the sections that the valves in the three trunks are not situated at the same level. Beginning from the point of origin of these trunks, the pulmonary artery is the first vessel provided with the valves, the next is that of the left systemic, and last of all is the valve of the right systemic.

HEART BEAT

The venous blood from the different parts of the body comes into the sinus venosus, the walls of which are feebly muscular and contractile. The contraction of the heart of Uromastix begins in the sinus venosus, which on contraction forces the blood into the right auricle through the sinu-auricular aperture. At the same time, the left auricle is filled with the arterial blood brought by the pulmonary vein from the lungs.

Muscular contraction starting from the sinus venosus spreads over the auricles, which are also contractile, and thus the blood is driven through the wide auriculoventricular apertures into the single ventricle. On the contraction of the ventricle the blood is allowed to go into the aortic arches only.

In spite of the undivided condition of the ventricle, a fair amount of double circulation takes place in the Uromastix, as in a completely divided ventricle. The ventricle is a spongy chamber with its cavity divided by muscular strands. When the heart beats, the auricles contract together, the right side of the ventricle receives (impure) blood from the right auricle, while the left side receives oxygenated blood from the left auricle. The spongy nature of the ventricle retards the mixing of these two qualities of blood to a great extent, except in the middle. The aortic arches arise a little toward the right of the ventricle (fig. 3 C), so when the ventricle contracts, the impure blood of the right side is first pumped out, then a portion of the mixed blood, and lastly the pure blood from the left side. Starting from the right side of the ventricle, the pulmonary arch arises first; next, the left systemic, and last of all the right systemic. The pulmonary artery arising from the right will therefore receive the impure blood of that side of the ventricle. The left systemic arising next will receive the mixed blood, and the right systemic arising last will get the blood which is pure.

ARTERIAL SYSTEM

The main source of our knowledge of the arterial system of Lacertilia is the works of Corti(6), written in 1847, "De systemate vasorum *Psammosauri grisei.*"

About the year 1857, Rathke(20) published a splendid paper, mainly dealing with the aortic arches, which was the result of a thorough examination of fifty-five different species of Lacertilia.

Hochstetter(13) and Zuckerkandl(28–30) described the arteries of the gut and those of the fore and hind limbs, respectively. Their observations are reproduced in Bronn's "Thierreich" by Hoffmann in his account of the Reptilia.

Beddard(1–5) has worked on comparative lines and has made studies in a number of different forms. O'Donoghue (18) gives a detailed account of the blood-vascular system of Sphenodon. Thapar(24), while describing the arterial system of Varanus bengalensis, has given brief notes on the arterial system of Uromastix. This is, in fact, the only work on this species, and this description is quite inadequate.

The presence of the semilunar valves at the base of the aortic arches has already been dealt with by the help of various sketches. All these trunks pass ventrad between the two auricles, rotating slightly in a clockwise direction.

The carotid arch (figs. 1, 6, and 8)

The two common carotids arise from the right systemic, in its anterior region, before it commences to bend outward. According to Rathke, various arrangements regarding the origin of carotids from the systemic might be seen in the

Lacertilia. The two common carotids might arise separately from the right systemic, or in some forms they come off from a common trunk arising from the systemic which is termed as carotis primaria by Rathke. It is the same thing as the innominate vein in the case of Mammalia. O'Donoghue (18) writes:

> Even when present the carotis primaria varies considerably in development reaching its maximum in the *Varanidae*, where it is quite long, and becoming so small in certain groups including the Lacertidae, that it may be overlooked.

In Uromastix, however, it is quite prominent.

The right carotid (figs. 6 and 8)

The common right carotid runs forward and outward for a short distance, where it divides into two branches. The two common carotids and their branches follow almost identical courses on the two sides of the neck and head, so the description given for one will apply equally well to the other. A. The external carotid. B. The internal carotid.

External and internal carotids are also considered as ventral and dorsal carotids, respectively. Both these vessels run forward along the lateroventral wall of the oesophagus.

A. The right external carotid (fig. 6)

This is a small artery arising from the outer wall of the common carotid. The point of origin of the external carotid, according to Rathke, is very close to the internal carotid, at a place where it receives the ductus caroticus. This very condition is seen in Uromastix. This vessel has a fairly wide distribution. Just near its origin it gives various arteries for the supply of the thyroid, trachea, shoulder and neck muscles, and then runs forward to the region of tongue and larynx, where it becomes divided into very small branches.

A. 1. *The anterior thyroid artery.* It is a small vessel arising from the external carotid close to its origin, which runs inward and supplies the anterior lateral lobe of the thyroid. Rathke mentions that in the Lacertilia this branch may sometimes come from the external or even from the common carotid, but he calls it thymus artery by mistake. In Sphenodon, too, this artery arises in a similar manner. Many small arteries arise close to the place of origin of the anterior thyroid artery.

A. 2. *The tracheothyroid artery.* The origin of this vessel is hidden from view by the anterior thyroid on the ventral side. This artery runs parallel to the anterior thyroid and soon becomes divided into two branches, supplying the oesophagus by one, trachea and thyroid by the other.

2. (a) *The oesophageal artery* arises at a considerable distance and runs anteriorly to supply the oesophagus.

2. (b) *The tracheothyroid artery* runs parallel to the anterior thyroid. As soon as it approaches the thyroid gland it divides into two branches. One proceeds anteriorly and is a comparatively larger branch supplying the thyroid and trachea, while the second branch supplies the thyroid.

2. (b) i. *The median thyroid artery* arises in the middle region of the glandular mass and runs inward for the supply of the central part of the thyroid.

2. (b) ii. *The tracheal artery* passes upward by the side of the trachea, to which it sends small twigs, finally breaking and terminating in it. This vessel passes up to the larynx, and in some specimens, where the injection mass could pass nicely, it has been seen supplying the larynx also. Its origin varies in different lizards. I have seen it arising from the pulmonary arch in Hemidactylus, and O'Donoghue, in Sphenodon, also describes its origin from the pulmonary. In the above-described

cases it supplies the larynx and is known as the laryngeal artery. Several branches run toward the dorsal side of the trachea also.

2. (b) iii. *The posterior thyroid artery* is a small vessel which breaks up into a series of small branches supplying the posterior lobe of the thyroid gland.

Both the anterior thyroid and the tracheothyroid arteries arise from the inner margin of the external carotid. There are other vessels which arise from the external border at that very place and supply the neck muscles, etc.

A. 3. *The cervicomuscular artery posterior* is a vessel which bifurcates near its origin and runs out to supply the posterior muscles in the region of the neck and the shoulders, near the attachment of the arm.

A. 4. *The buccal artery* arises independently from the external carotid. It bifurcates and supplies the floor of the buccal cavity.

A. 5. *The cervicomuscular artery anterior* is another vessel arising from the external carotid for the supply of neck and shoulder muscles in the anterior region. It is near the posterior margin of the head.

The external carotid then runs forward, and in the posterior region of the head shows a slight curvature. It runs a short course and then gives rise to a big vessel for the supply of the floor of the mouth, hyoid, larynx, and trachea. The branch for the floor of buccal cavity is very small, and the main supply, therefore, is for larynx and trachea.

A. 6. *The laryngeal-tracheal artery.* This is a fairly large branch arising from the external carotid, for the supply of trachea and larynx. It sends a small branch for the supply of the anterior region of the buccal floor.

A. 6. i. *The buccal artery anterior* is a small branch which divides into a series of small vessels supplying the buccal cavity in its anterior region.

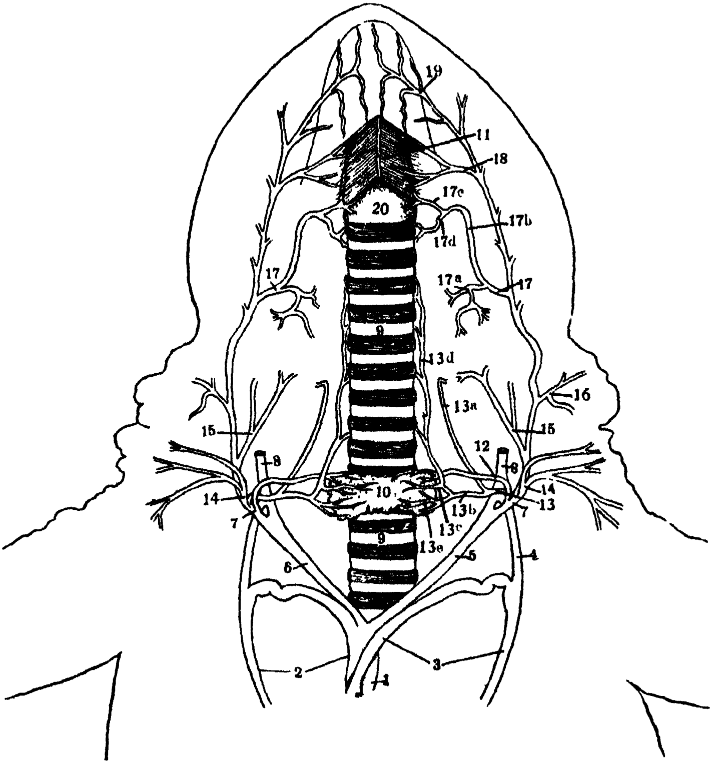


Fig. 6 The external carotid.

1, pulmonary arch
2, right systemic arch
3, left systemic arch
4, ductus caroticus
5, left common carotid
6, right common carotid
7, external carotid
8, internal carotid
9, trachea
10, thyroid gland
11, genioglossus muscle
12, anterior thyroid artery
13, tracheothyroid artery
13a, oesophageal artery
13b, tracheothyroid
13c, median thyroid
13d, tracheal artery
13e, posterior thyroid
14, cervicomuscular artery posterior
15, buccal artery
16, cervicomuscular artery anterior
17, laryngeal tracheal artery
17a, anterior buccal
17b, laryngeal tracheal artery
17c, laryngeal artery
17d, tracheal
18, genioglossal artery
19, submandibular artery
20, larynx

A. 6. ii. *The laryngeal-tracheal artery* runs parallel with the external carotid. Near the region of the larynx it bifurcates, supplying the larynx and the anterior portion of the trachea.

ii. (a) *The laryngeal artery* is a small vessel which breaks up into a number of small twigs supplying mainly the larynx and partly the genioglossus muscles, which hide it from view on the ventral side.

ii. (b) *The tracheal artery* is another small vessel for the supply of the anterior portion of the trachea. This vessel runs toward the dorsal side of the trachea, where it breaks up into small twigs.

The external carotid, which becomes very much reduced by this time, furnishes, as it ascends, a number of small vessels to the muscles of the lower jaw.

A. 7. *The genioglossal artery* arises from the external carotid at the level of the larynx. It runs forward and inward along the side of the genioglossus muscle, to which it gives branches supplying the ventral side of the muscle.

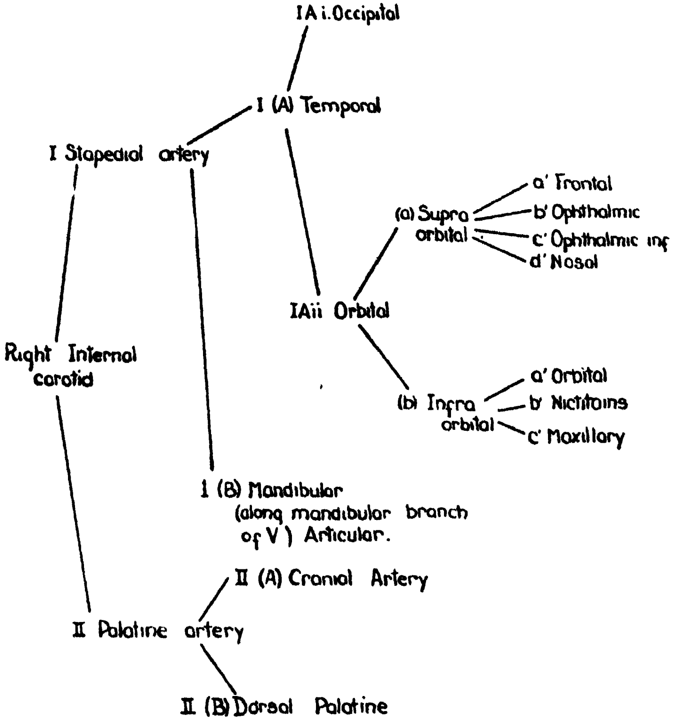
The external carotid proceeds forward, giving branches to the adjacent muscles throughout its course. The terminating point of the carotid is termed as—

A. 8. *The submandibular artery*, which runs forward to the anterior end up to the place of symphysis. This vessel becomes very widely distributed for the supply of hyomaxillary and hyoidean muscles. Some branches run posteriorly for the supply of the anterior portion of the genioglossus muscles.

It supplies the tongue, hyoid, and various inner muscles of the mandible. It ends behind the mandibular symphysis.

Right internal carotid (fig. 7)

This is a fairly large branch at least twice the diameter of the external carotid. From the point of origin it passes slightly outward. A little beyond, it receives the ductus caroticus, and then it turns forward along the lateral margin of the quadrate, giving small twigs to the neck muscles during its course. It supplies the whole of the face, jaws, and brain. In the outer and posterior margin of the auditory capsule it bifurcates. The course and the arrangement of the chief vessels may be represented diagrammatically as follows:



The two branches into which the internal carotid bifurcates are known as the, I) stapedial artery and the, II) palatine artery.

I. The stapedial artery

The stapedial artery has been termed as carotis externa and artery facialis by Corti and Rathke, respectively. O'Donoghue calls this the stapedial. It is a comparatively larger vessel than the palatine, and its ramifies the whole of the occipital, temporal, orbital, a considerable part of the maxillary, and the mandibular regions. In the posterior margin of the auditory rim it bifurcates, one branch, the *temporal artery*, ascending and running dorsally over the auditory capsule, and the second, the *mandibular artery*, descending and running in a short curve along the posterior ventral border of the capsule.

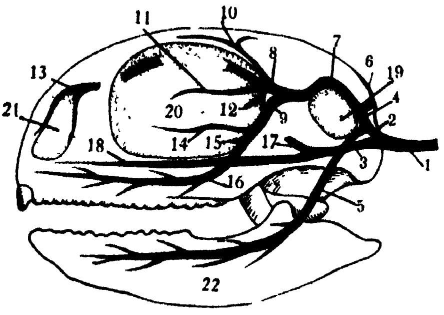


Fig. 7 The internal carotid.

1, internal carotid
2, stapedial artery
3, palatine artery
4, temporal artery
5, mandibular artery
6, occipital artery
7, orbital artery
8, supra-orbital artery
9, infra-orbital artery
10, frontal artery
11, ophthalmic artery
12, ophthalmic inferior artery
13, nasal artery
14, orbital artery
15, nictitating artery
16, maxillary artery
17, cranial artery
18, dorsal palatine artery
19, auditory capsule
20, orbit
21, olfactory capsule
22, lower jaw

I (A) *The temporal artery* is slightly the larger of the two branches into which the stapedial divides.

After giving a small branch I (A) i, *the occipital artery,* it is continued as I (A) ii, *the orbital trunk,* which then curves over the auditory capsule and runs up to the posterior margin of the orbit, where it becomes divided into several branches supplying the frontal, orbital, and the nasal regions.

I (A) i. *The occipital artery* is a small vessel for the supply of the muscles in the occipital region.

I (A) ii. *The orbital artery* is a large vessel, which at the posterior end of the orbit further becomes divided into two branches, one, ii (a), running along the upper border of the orbit, and another, ii (b), below the orbit toward the ventral side of the jaw.

I (A) ii (a) *The supra-orbital* (Rathke) was termed by Gaupp the orbitonasalis, on account of its distribution. This vessel will be visible on the removal of the eyeball. It occupies a dorsal position. Near the point of origin, i.e., at the posterior end of the orbit, it gives off the following three branches:

a′. *The frontal artery* is a small vessel arising behind the orbit. It ascends upward to the frontal bone, where it becomes divided for the supply of muscles in that region.

b′. *The ophthalmic artery* is a somewhat larger branch running in the central region of the orbit, toward the back of the eyeball. It is distributed to the muscles of the eyeball.

c′. *The ophthalmic inferior* arises near the one described above. It supplies the eyeball and ultimately disappears by small capillaries into the eye muscles.

The main vessel, *d′*, the supra-orbital, then continues forward in the same dorsal position along the wall of the orbit. It passes through the internasal septum, and after running a short distance it reenters the orbit at its anterior end. This branch of the supra-orbital may be called the *nasal artery*. It enters toward the dorsal side of the olfactory capsule, bifurcates, forming two main twigs, the anterior and the posterior, which become distributed to the olfactory organ and the mucous membrane and send small branches to the subcutaneous tissue of the snout.

I (A) ii (b) *The infra-orbital* is the second branch of the orbital. It leaves the temporal at the posterior border of the orbit, and runs sharply downward. It soon follows a forward course, running on the floor of the orbit along with the orbital branch of the trigeminal nerve. It furnishes one or two branches in the orbital region and then continues forward as the maxillary artery.

a′. *The orbital artery* arises from the infra-orbital as it passes through that region. It supplies some of the tissues in the orbit.

b′. *The nictitating artery* is another vessel arising from the infra-orbital for the supply of the nictitating membrane.

c′. *The maxillary artery* is, in fact, the main continuation of the infra-orbital. It becomes distributed to the maxilla. It runs parallel with the jaw, entering the substance of the maxilla and terminating in the subcutaneous tissues of the snout. During its course it gives off a number of small branches supplying the upper lip.

I (B) *Mandibular artery.* The mandibular artery, as mentioned above, runs downward from the stapedial and follows the same course as the mandibular branch of the fifth nerve. It enters the bone through the articular foramen and continues forward in the bone, terminating at the symphysis. Various arteries are given off from it to the tissues of the lower jaw.

II. Palatine artery

The palatine artery supplies the roof of the mouth and the brain.

II (A) *The cranial artery* is a fairly large branch arising from the palatine for the intercranial distribution.

II (B) *The dorsal palatine* is the main trunk which runs forward, beneath the palatine bone. It runs more or less parallel to the maxillary branch. It gives off numerous small branches to the roof of the mouth, breaking and finally terminating in the subnasal region.

THE SYSTEMIC ARCHES

The right and left systemic arches come off separately from the ventricle, as has been mentioned above. Both these receive on their anterior walls the ductus caroticus (admitted by Thapar). They then curve round the oesophagus to pass backward and inward, thus becoming dorsal in position, and finally run back to unite with each other in the middle line below the vertebral column, behind the level of the heart, to form the dorsal aorta. At a considerable distance before the two systemics unite to form the dorsal aorta, there arises from the right systemic a common subclavian artery, about an inch long, which mainly divides into right and left subclavians, running on either side to supply the arm and its muscles.

The origin of the subclavian varies in different lizards. Beddard ('05) has pointed out that this artery actually arises from the right systemic, but just at a point where it joins the left. In Uromastix, however, it arises long before the union of the two systemics. Thapar has rightly indicated its position in the sketch. Both the subclavian arteries pass outward, each entering the arm and dividing into the following branches supplying the various parts of the shoulder-girdle:

a. The scapular artery supplies the scapular region.

b. The coracoid artery is distributed to the muscles of the coracoid.

c. The brachial artery is the median vessel which is the main branch of the subclavian entering the arm, and supplying the various muscles of the arm and forearm, issuing branches to the carpals.

I have not been able to see the epigastric artery, which has been described by O'Donoghue as arising from the subclavian. Several injections were made with the hope of demonstrating its presence, but I have to confirm the observation of Thapar, who has described at length an epigastric arising from the common carotid in Varanus, and he also says that he has not been able to trace any vessel corresponding to the epigastric of Varanus in Uromastix. He further adds that in vertebrates the epigastric is generally described as originating from the subclavian. The origin of an epigastric artery supplying the liver, fat-body, and body wall from the root of the common carotid is very peculiar. It is not found in any of the Lacertilia, and he is unaware of any similar vessel in any other vertebrate.

At the place where the common subclavian bifurcates to form the right and left subclavians arises a median artery which runs forward along the right side of the cervical region of the vertebral column. No mention of this artery has been made by Thapar. He has, however, described a pair of vertebral arteries in Varanus where both of these arise from the subclavians after their division into right and left. In Uromastix the right vertebral arises as described above, while the left takes its origin from the subclavian of that side—a condition similar to that of Varanus. Both the vertebrals run along the sides of the cervical vertebrae. So there is a pair of these vertebrals, both with different origins and supplying all the six neck vertebrae and muscles anteriorly, and posteriorly giving branches to some of the anterior dorsals, sending out a pair in the posterior portion of every vertebra.

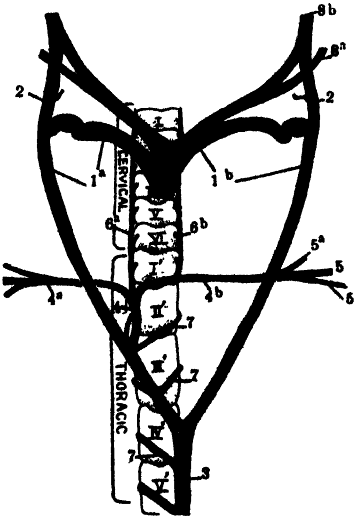


Fig. 8 The systemic arches.

1a, right systemic arch
1b, left systemic arch
2, ductus caroticus
3, dorsal aorta
4, common subclavian artery
4a, right subclavian
4b, left subclavian
5, brachial artery
5a, scapular artery
5b, coracoid artery
6a, right cervical artery
6b, left cervical artery
7, parietal artery
8a, external carotid
8b, internal carotid
I, II, III, IV, V, VI, cervical vertebrae
I', II', III', IV', V', thoracic vertebrae

In order to locate the right position of the origin of various arteries from the systemic arch and the dorsal aorta, it is best to know a small portion of the skeleton of the animal, particularly the vertebral column. It is divisible into five different regions, the cervical, the thoracic, the dorsolumbar, the sacral, and the caudal, each consisting of 6, 5, 13, 2, and 25 to 27 vertebrae, respectively.

The parietal arteries (segmental arteries) are paired branches supplying the muscles of the dorsal region and the body wall. They arise in regular order in front of each vertebra.

The muscles in relation to the first six vertebrae get their supply from the vertebral. First thoracic or the dorsal also gets a pair of arteries from the vertebrals. This is, in fact, the first pair which is given off by the vertebrals close to their origin. Between the origin of the subclavian and the union of the two systemics arise two pairs of vertebral arteries supplying the second and third thoracic vertebrae. The last two thoracic vertebrae (fourth and fifth) receive direct supply from the dorsal aorta.

The origin and the arrangement of the parietal vessels may be represented diagrammatically as in the table (p. 306).

The left systemic (figs. 8 and 9)

As it arises from the ventricle it proceeds forward, where it receives on its anterior wall the ductus caroticus in the same manner as the right arch. It also becomes dorsal like the latter and runs along the inner border of the lung. A small oesophageal artery (fig. 9) is the only vessel arising from this arch. In some specimens a pair of these oesophageal arteries was seen. In many lizards several vessels are given off before the left and right arches unite. As described by Thapar in Varanus, the main arteries supplying the gut (mesenteric and gastric arteries) arise definitely from the left arch. In fact, in such a case these vessels carry a major portion of the blood to the gut, and the main arch thus becomes very much reduced before it unites with the right arch to form the dorsal aorta. It is an important item regarding the difference in origin of the visceral arteries in various lizards.

Thapar says:

> In the origin of the mesenteric artery from the left systemic arch we find in Varanus, a condition which resembles that of higher

reptilia, and which differs from that of other genera of Lacertilia such as *Heloderma*, *Uromastix* and *Hemidactylus* where all the visceral arteries arise from the dorsal aorta after the union of the two systemics.

ARISING FROM	NO. OF VERTEBRAE	ORIGIN OF PARIETALS	VARIOUS ARTERIES AND THEIR DISTRIBUTION
Vertebral	1	Cervical	Muscles of the neck
Vertebral	2	Cervical	Muscles of the neck
Vertebral	3	Cervical	Muscles of the neck
Vertebral	4	Cervical	Muscles of the neck
Vertebral	5	Cervical	Muscles of the neck
Vertebral	6	Cervical	Muscles of the neck
Vertebral	7	Thoracic	Thoracic muscles
Systemic arch right	8	Thoracic	Thoracic muscles
Systemic arch right	9	Thoracic	Thoracic muscles
1. Dorsal aorta	10	Thoracic	Oesophageal
2. Dorsal aorta	11	Thoracic	Gastric anterior
3. Dorsal aorta	12	Lumbar	Gastric posterior
4. Dorsal aorta	13	Lumbar	Gastric medius
5. Dorsal aorta	14	Lumbar	
6. Dorsal aorta	15	Lumbar	Posterior mesenteric
	Between 15 and 16		Coeliac
7. Dorsal aorta	16	Lumbar	
	Between 16 and 17		Pancreohepatic
8. Dorsal aorta	17	Lumbar	Anterior mesenteric
9. Dorsal aorta	18	Lumbar	
10. Dorsal aorta	19	Lumbar	Spermatic-ovarian
	Between 19 and 20		Suprarenal
11. Dorsal aorta	20	Lumbar	
12. Dorsal aorta	21	Lumbar	
13. Dorsal aorta	22	Lumbar	
	Between 22 and 23		Rectal
14. Dorsal aorta	23	Lumbar	
15. Dorsal aorta	24	Lumbar	Iliacs and rectal

It is, of course, a fact that the quality of blood carried by the two systemics is slightly different. The right systemic contains the pure blood, which goes to the head region. The left systemic carries the mixed blood. In the Varanus type it is then the mixture which goes to the different regions of the gut, as the main vessel arises from the left systemic. In such a case compensation is afforded by an epigastric which is given off from the innominate and carries the pure blood. In Heloderma, Uromastix, and Hemidactylus types these vessels arise from the dorsal aorta, i.e., after the union of the two systemics. When the two arches unite, surely pure blood is added to the mixture in the aorta. Also the presence of the ductus caroticus in such animals tends to reduce the amount of venous blood in the left systemic by the addition of arterial blood from the carotids. The first rush of blood from the ventricle is into the right systemic and innominate. The ductus caroticus performs a very important function in equalizing the pressure and the quality of blood in the arches.

THE DORSAL AORTA (fig. 9)

The two systemics unite in the middle region of the lung to form the dorsal aorta. All the main branches supplying the alimentary canal and the remaining visceral organs arise independent of one another. No such combination of a single vessel arising for the supply of the gut as seen in Varanus is found in Uromastix.

The dorsolumbar or the parietal arteries

After the union of the two systemics there arise about fifteen arteries from the dorsal aorta, throughout its course as far as the appearance of the tail, where the dorsal aorta terminates and is known as the caudal artery. These are the dorsolumbar or the parietal arteries arising from the dorsal side of the aorta and at once bifurcating to supply both sides of the vertebral column, each pair entering the dorsal muscles at the posterior end of each vertebra. Out of these fifteen pairs, the first two supply the thoracic vertebrae and the remaining thirteen are the lumbars. It will be in relation to these parietals that the position of the other (visceral) arteries arising from the aorta will be described.

1. *The oesophageal artery* is the first vessel arising from the dorsal aorta in front of the first parietal (fourth thoracic). It is a small vessel running and branching on the sides

of the oesophagus. Thapar makes no mention of it. In Sphenodon the oesophageal arises from the systemic arch, similar to one described above. In Uromastix, therefore, it is the second oesophageal.

2. *The gastric arteries* are about three in number, all arising independently from the dorsal aorta for the supply of the various regions of the stomach.

2 a. *The anterior gastric artery* leaves the dorsal aorta about the level of the second parietal (last or fifth thoracic) and supplies the anterior end of the stomach.

2 b. *The posterior gastric artery* is a large branch arising close to the origin of the anterior gastric. It immediately divides into several branches supplying the posterior region of the stomach.

2 c. *The medius gastric artery* is also a fairly large branch arising about the level of the fourth parietal (second lumbar). It divides into several branches supplying the midregion of the stomach.

Thapar has made no mention whatsoever of the anterior and posterior gastrics. He has shown a gastric, no doubt, in the sketch, which seems to correspond to the gastric medius. It has been represented by him as the only artery supplying the stomach.

3. *The posterior mesenteric artery* is the next to arise from the dorsal aorta, at the level of the sixth parietal (or fourth dorsolumbar), and is to be described later.

4. *The coeliac artery* (or superior mesenteric, Beddard) is a very large artery arising from the dorsal aorta between the sixth and seventh parietals. It is mainly concerned with the supply of the stomach, issuing branches to the pancreas and spleen also. From the point of its origin it runs as a single vessel for a short distance, and then bifurcates, one branch being purely for the supply of the stomach. It ramifies all over the dorsal region of the stomach, giving off a small artery to the anterior region of the spleen. The second branch supplies the stomach, spleen, and pancreas. It takes the following course:

4 a. The anterior branch gives off a small splenic artery supplying the anterior portion of the spleen. The main artery is a large vessel, supplying the dorsal region of the stomach.

4 b. The second or posterior branch is a large artery. It furnishes a pair of splenic arteries for the main supply of the spleen in its posterior region, and then the main artery curves back along the inner wall of the stomach, supplying innumerable branches to it. It bifurcates posteriorly. One limb continues backward as the pyloric artery supplying the pyloric end of the stomach, while the other artery curves forward and outward over the ventral wall of the stomach and gives several branches to it. This branch has been wrongly described by Thapar. He calls this vessel the pancreohepatic and describes its distribution in the following words:

> The Pancreo-hepatic which runs along the pancreas (Pa) sending branches to this organ as it proceeds, curving over the stomach to supply the liver as the hepatic artery (he).

No artery from this branch proceeds to the liver in Uromastix. In Sphenodon, however, the hepatic artery derives its origin from this branch, and it leaves the main vessel just before it runs on to the stomach wall and enters the liver more or less ventrally, as described by Thapar.

It appears Thapar has confused the account of Sphenodon with that of Uromastix, without determining this point more carefully.

5. *The pancreohepatic* is a fairly big branch arising from the dorsal aorta about the level of the seventh or eighth parietal. This vessel has a fairly wide distribution. It runs as a single trunk for a considerable distance, but before reaching the region of the duodenum it divides into three branches supplying, *a*) the duodenum, *b*) the liver, and, *c*) the pancreas.

5 a. *The duodenal artery* is the most anterior branch arising from the pancreohepatic. It curves forward and supplies the distal portion of the duodenum.

5 b. *The hepatic artery* is the central branch which lies under the proximal part of the duodenum, and after giving off a pair of duodenal arteries, it runs forward and becomes

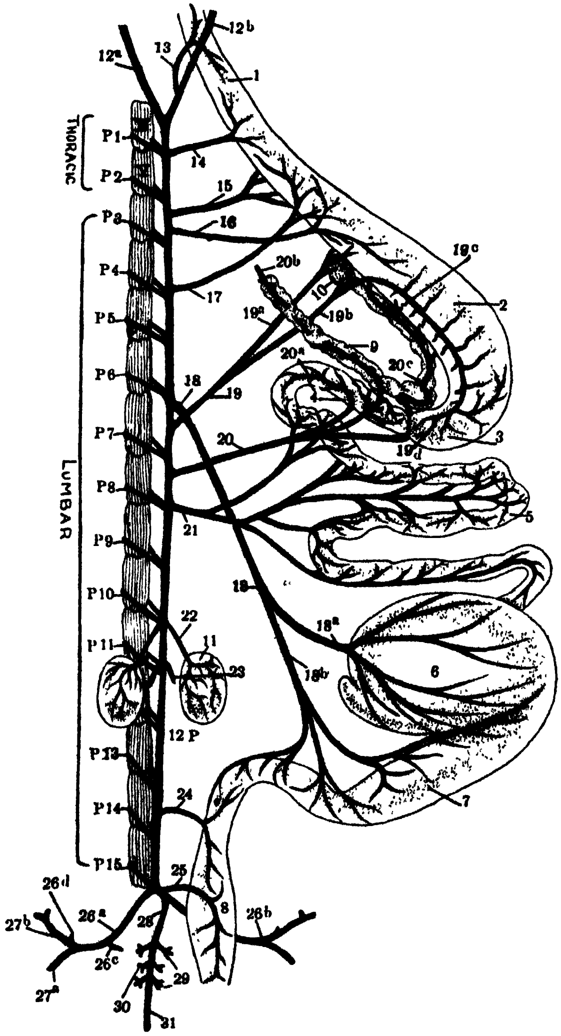


Figure 9

embedded in the pancreas, till it enters the left lobe of the liver as the hepatic artery, giving off small pancreatic arteries during its course. The hepatic artery on entering the left lobe of the liver is lost in it, but in the injected specimens it is seen again emerging out of this lobe, and breaking into several branches supplying the gall bladder and the right lobe of the liver.

5 c. *The pancreatic artery* is the third branch of the pancreohepatic trunk. It loops around and passes dorsally through the groove produced by the beginning of the duodenum, and at once enters the pancreas. It then becomes embedded in this gland and runs forward in the limb of the pancreas which extends along the inner concave border of the stomach. It furnishes several branches to the gland throughout its course and also supplies the wall of the stomach.

Fig. 9 The dorsal aorta.

1, oesophagus
2, stomach
3, pyloric end of stomach
4, duodenum
5, small intestine
6, caecum
7, colon
8, rectum
9, pancreas
10, spleen
11, testes
12a, right systemic arch
12b, left systemic arch
13, oesophageal no. 1
14, oesophageal no. 2
15, anterior gastric
16, posterior gastric
17, medius gastric artery
18, posterior mesenteric artery
18a, caecal artery
18b, colon artery
19, coeliac artery (superior mesenteric)
19a, splenic artery
19b, splenic artery
19c, gastric artery
19d, pyloric artery
20, pancreohepatic artery
20a, duodenal artery
20b, hepatic artery
20c, pancreatic artery
21, anterior mesenteric
22, spermatic
23, external spermatic artery
24, rectal artery
25, cloacal artery
26a, right common iliac
26b, left common iliac
26c, hypogastric
26d, pelvic artery
27a, external iliac
27b, internal iliac
28, renocaudal
29, renal arteries
30, renocloacal artery
31, caudal artery
P.1, 2, parietals (thoracic vertebrae IV, V)
P.3–15, parietals (lumbar)

It will thus be seen that this artery forms a very important part of the visceral system and supplies the various organs of the body extensively.

Thapar has described this pancreohepatic as the duodenal, and has mentioned it as a small branch supplying the duodenum only.

6. *The anterior mesenteric* is a large vessel arising from the dorsal aorta in the region of the eighth parietal. The main trunk is for the supply of the small intestine. It divides into a series of four or five large branches which become further distributed to the various regions of the intestine. Thapar calls this the intestinal.

3. *The posterior mesenteric* is also a large artery for the supply of the lower parts of the intestine. The origin of this vessel has already been described from the dorsal aorta at the level of the sixth parietal. As it supplies the posterior region of the gut, its description has accordingly been taken here at the right place. From the place of its origin this vessel runs back ventrally over all the arteries described above arising from the aorta, and it divides into two branches.

3 a. *The caecal artery* is the only supplying the caecum. It gives off branches at the junction of the intestine and the caecum.

3 b. This branch is for the colon. This becomes further distributed into two or three vessels supplying the distal region of the caecum and the whole of the colon.

7. *The spermatic arteries.* There is a pair of these arteries arising near the level of the tenth parietal. These vessels run back to the testes on both sides. Some branches of the spermatic supply the suprarenal bodies situated dorsad to the testes. This resembles the distribution of the ovarian vessels. Close behind the spermatic arise two small arteries which supply the vasa deferentia or the oviduct, as the case may be.

8. *The rectal artery* arises far down between the thirteenth and fourteenth parietals, and passes down along the dorsal wall of the rectum. In front it runs up to the region supplied

by the posterior mesenteric. In Sphenodon, O'Donoghue mentions that actual anastomosis takes place between rectal and the posterior mesenteric branches. I have not been able to find this condition in Uromastix.

9. *The cloacal artery* arises at the fifteenth parietal, at the spot where the two iliacs arise. It passes dorsal to the wall of the rectum and is distributed to the lateral walls of the cloaca and extreme end of the rectum. Posteriorly, it gives off a branch supplying the margins of the cloaca.

10. *The common iliac arteries* arise at the level of the last or the fifteenth parietal pair, before the anterior margin of the sacral region of the vertebral column. There is a pair of these which are mainly for the supply of the hind limbs. They run tangentially to the anterior border of the kidney. The branches arising from this common trunk are:

10 a. *The hypogastric artery* arises from the common iliac close to the region of the kidney. It is a small vessel which ultimately supplies the urinary bladder.

10 b. Near the base of the limb is given off a branch anterior to the muscles of the proximal end of the thigh.

10 c. *The pelvic artery* is a small vessel supplying the pelvic girdle and the fat-body.

The main vessel (the common iliac) becomes divided into: (10) i. The internal iliac; (10) ii. The external iliac.

(10) i. *The internal iliac* runs along the ischiadic nerve, and it is on account of its relation to this nerve that it is described as sciatic artery in many text-books (O'Donoghue). It is distributed for the supply of various muscles of the thigh.

(10) ii. *The external iliac* is the main artery supplying the various muscles of the hind limbs. It runs along the whole length of the limb. Thapar's account and his figure 4 showing this region are not at all intelligible. He has described and shown a pair of ischiadic arteries (*is*) arising from the dorsal aorta behind the fifteenth pair of parietals, each giving off a branch to the posterior epigastric (*p.e.*), which runs forward to the body wall, ultimately terminating in the fat-

body. This is, in fact, the iliac, which has wrongly been described by him as the ischiadic. The ischiadic is only a branch of the iliac, and it cannot, therefore, have a direct origin from the dorsal aòrta.

The aorta, after giving off the iliacs, continues backward as the renal and the caudal vessels. It should ordinarily run in the middorsal line, at the place where the two iliacs arise, but its position in the case of Uromastix is slightly asymmetrical, in the sense that the whole of the dorsal aorta at the level of the fifteenth parietal appears to be bifurcated into two common iliacs, and it is later from the left iliac trunk that the renal and caudal vessels arise. Close to its origin it turns to the right in order to maintain the central position before giving rise to the renals.

11. *The renal arteries.* The renocaudal vessel enters the kidney (which is fused behind and free in front, therefore Y-shaped, and situated right at the base of the tail, as is made evident by dissecting a portion of the tail), where it gives off two or three pairs of vessels running into its substance.

12. *The renocloacal artery* (O'Donoghue) arises at the level of the midregion of the kidney, from the dorsal aorta.

It becomes divided into several small branches supplying the posterior margins of the cloaca.

The dorsal aorta runs into the substance of the posterior fused portion of the kidney, from which it is seen emerging posteriorly.

13. It then passes into the haemal canal of the caudal vertebrae, and is known as the caudal artery, giving off small paired parietal arteries to the muscles of the tail. Thapar has again wrongly shown iliacs arising from the dorsal aorta posterior to the renals. As already stated, the kidneys in the case of Uromastix lie far behind, quite a considerable distance posterior to the hind limbs. The iliacs, therefore, cannot possibly arise at a place shown by him.

SUMMARY

The arterial system of Uromastix hardwickii retains many primitive features and shows great resemblance and relationship to that of Sphenodon. The heart is typically reptilian. The conus arteriosus is absent. Two systemics and a pulmonary arch arise from the ventricle. The carotids arise together from the right systemic by means of a small common carotis primaria. A very well-developed ductus caroticus connects each carotid with the systemic arch of its side. The ductus arteriosus (ductus Botalli) is absent. The dorsal aorta is formed by the union of both the systemic arches. The left systemic joins nearly entire, while the right one, which is termed the systemicocarotid trunk, furnishes the carotids, the vertebral, a single subclavian, and two pairs of parietal arteries before union. The epigastric arteries do not arise from the carotids and are altogether absent. The dorsal aorta gives off fifteen pairs of parietal arteries which are segmentally arranged.

It is very remarkable that all the main branches supplying the alimentary canal and the remaining visceral organs arise independently of one another, there being no such combination as is found in Varanus, etc.

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SEASONAL CYCLES OF THE INTERSTITIAL CELLS IN THE TESTIS OF THE HORNED TOAD (PHRYNOSOMA SOLARE)

SEASONAL VARIATIONS IN THE NUMBER AND MORPHOLOGY OF THE INTERSTITIAL CELLS AND THE VOLUME OF THE INTERSTITIAL TISSUE

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FIVE TEXT FIGURES AND ONE PLATE (FOUR FIGURES)

AUTHOR'S ABSTRACT

A histological study has been made of a series of Phrynosoma testes taken at frequent intervals throughout the year. As it was important to differentiate between the amount of interstitial tissue and the number of cells, it was necessary to take into account the percentage of tissue, total testis volume, and the number of cells per unit volume.

The results show a definite interstitial-cell cycle. Volume of interstitial tissue and size of individual cells are greatest during the breeding season, but the interstitial cell number is then minimal. The maximal number of interstitial cells occurs after the close of the breeding season. There is, apparently, no reversion of interstitial cells to a connective-tissue-cell type.

The above changes are correlated with the spermatogenetic cycle and with increases in total tubule length, in tubule diameter, and in testis volume. The time in which important changes in ovarian volume occur and at which ovulation takes place is coincident with the testicular changes.

Interstitial cells have been stated to have no endocrine function for the reason that their number is at a minimum during the breeding season. If, however, interstitial tissue is responsible for sex characters and activity, the present work would indicate that it is the volume of tissue, and not the number of cells, that is important.

INTRODUCTION

Although there is no doubt that the male gonad produces a hormone, it is still uncertain which particular testicular tissue is responsible for its secretion. Some workers have assigned this function to the interstitial cells, but the evidence is at present insufficient and there is much which is contradictory.

The embryonic relationships of these cells in free-martin gonads have been studied by Lillie ('17, '23), Chapin ('17),

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[1] The preparation and study of material were done at the University of Arizona. The manuscript has been prepared in the Osborn Zoölogical Laboratory, Yale University.

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Lillie and Bascom ('22), and Bascom ('23). These studies have shown a relationship between the development of the interstitial cells of the male twin and the abnormal development of the sex organs of the female.

There is a group of experimental procedures which has as its object the elimination of various testicular elements, and thus permits the study of interstitial cells alone. Such is the case with x-ray treatment by Benoit ('24), Regaud ('10), Barratt and Arnold ('11), and others. Oslund and Bachman ('26), however, show that the germinal epithelium may not be completely destroyed by x-ray, and that it remains as a possible producer of sex hormones.

Experimental cryptorchidism reduces germinal epithelium, as shown by Moore ('24 a, b), Oslund ('26), and others. Fukui ('23 a) has shown that heat applied to the testis causes degeneration of the germinal epithelium. He believes that the cryptorchid results are probably due to abdominal temperature (Fukui, '23 b). Moore ('24 c, d; '26) has enlarged upon this work and considers the scrotum a thermoregulator. Animals with undescended testes have also been studied. Although they are generally sterile, their sex behavior is normal, and for this reason the relationships of the interstitial cells are of interest.

All of these procedures are subject to the criticism that destruction of germinal cells may not be complete.

If the interstitial tissue of the testis controls sex characters and activity, there should be a definite correlation between changes in this tissue and the appearance and disappearance of the breeding season. With this possibility in mind, studies have been made of the seasonal relationships of the interstitial cells and the spermatogenetic cycles.

Most of this work has been done upon the Amphibia. For the frog, the work of Champy ('24) and of Mazzetti ('11) indicates that the maximum development of the interstitial tissue comes after the breeding season. They conclude that the interstitial cells have nothing to do with the sex characters. The same conclusion is reached by Humphrey ('21) for Necturus. Aron ('24 a, b), however, found a definite relationship between the secondary sexual characters and the development of interlobular tissue of the testis both for Rana temporaria and for Rana esculenta.

The work on the mammals is contradictory. Tandler and Gross ('11) found that in the mole, Talpa europea, the interstitial cells were at their greatest development after the breeding season. Rasmussen ('17) reports a similar condition in the woodchuck, Marmota monax. Marshall ('11), however, reports the greatest interstitial-cell development during the breeding season in the hedgehog, Erinaceus europeas.

A complete review of the work on seasonal modifications has been made by Oslund ('28). The above brief review emphasizes the fact that the function of the interstitial cells and the source of testicular hormones are extremely problematic.

No seasonal studies upon reptiles have been made in this connection. Mazzetti ('11) has shown the interstitial cells to be abundant in the testis of a hibernating snake. They have been shown to be present in Testudo orbicularis by Pellegrini ('25) and in various species of Lacerta by Franckenberger ('22), Reiss ('23), and by Pellegrini ('25).

The present study of the testis of the horned toad, Phrynosoma solare, has been made in order to determine whether there are seasonal variations in the volume and character of the interstitial tissue, and if so, to determine their relationship to the spermatogenetic cycle, and also to correlate such testicular cycles with the ovarian cycle.

I wish to express my appreciation to G. T. Caldwell, under whose direction this work was undertaken, to Dr. D. B. Young and Ethel G. Stiffler for their assistance, and to Dr. J. S. Nicholas for criticism of the manuscript.

MATERIALS AND METHODS

The horned toad was chosen because of its several advantages for this type of work. In the first place, possible errors due to temperature changes are eliminated, as this lizard is a cold-blooded animal with abdominal testes. Fukui ('23 b) and Moore ('24 c, d; '26) have stated that temperature is an important factor in the regulation of cellular activity within the testis. In some warm-blooded animals the position of the testis varies from abdominal to scrotal with the seasons, and the temperature of the testis must vary. In such animals there is a possibility that the effects, which have been described, may be due, not to interstitial-cell influence, but to temperature changes.

The horned toad has one definite breeding season each year. Since the spermatogenetic cycle covers four months, the progressive and regressive stages of spermatogenesis are very widely separated. This is important, for in some of the higher animals the production of spermatozoa is continuous, and a seasonal study is of little value.

The testis of the horned toad resembles that of the mammal in the arrangement of the seminiferous tubules and the presence of a definite epididymis. The testis is small and can be sectioned entire, thus avoiding cutting and mutilation.

The testis responds to cyclic changes as a whole without any regional differentiation; that is, not only an entire tubule, but all the tubules are at the same stage of development. More than a thousand slides have been examined, and no differences in structure or development were noted between parts of the same testis.

Regional differentiation is prominently shown in some of the lower forms. Aron ('24 b) states that the interstitial cells are localized, at certain seasons, in definite areas of the testis of Rana esculenta. In some species of urodeles regional differentiation is great and even causes external constrictions and enlargements (Humphrey, '21, '22, '25). Humphrey ('21) looks upon testes with this 'caudocephalic movement' as favorable material for showing relationships between the spermatogenetic and interstitial-cell cycles. It might, however, be considered a decided disadvantage. Humphrey ('21) draws conclusions from the presence or absence of interstitial cells about a given lobule and their association with the spermatogenetic cycle within that lobule, but disregards the possibility of their presence in the remainder of the testis. There is some chance of error in such an interpretation of successive development stages within a single testis. If the tissue elements which have to do with hormone production are present and active at all, the part of the testis in which they are located is of little importance, since the hormone produced is distributed by the blood. The uniformity of structure of the horned-toad testis eliminates this source of error.

Although there are several species of the genus Phrynosoma found within the region of collection, the animals used have been very carefully limited to the one species, P. solare. All specimens were secured near Tucson, Arizona, and thus any variations due to climatic differences have been avoided.

During the summer months the horned toads were taken directly from the desert. For the winter months animals were allowed to hibernate naturally, by burying in the sand of an outdoor pen. At regular intervals male and female animals were killed and the gonads taken. In March, during the breeding season, and in September and October, several male and female animals were killed each week, and from November to February the interval between killings did not exceed two weeks. A brief summary of the material used, with collection dates, follows:

Months	*Males*	*Females*	*Months*	*Males*	*Females*
January,	6	6	August,	19	19
February,	4	7	September,	15	15
March,	11	14	October,	17	16
April,	17	20	November,	6	6
May,	23	23	December,	5	9
June,	28	28		——	—
July,	23	23	Total,	174	186

This collection of gonads gives a more complete series than any found in the literature, as three times the number of testes previously recorded have been studied. Body measurements were taken, and immature, deformed, diseased, or otherwise abnormal animals were very strictly rejected. The adult males averaged 13 cm. in length and 5.1 cm. in width, while the females averaged 13.3 × 5.6 cm. These figures were used to eliminate the immature animals. Size and volume measurements were recorded for all gonads at the time of killing. These measurements were taken to the nearest ½ mm. The volume of the ovaries was determined by liquid displacement. The volume of each testis was calculated from its length and diameter by considering the testis a perfect ellipsoid. Liquid displacement was not used for the testes, as that would have required the removal of the epididymis from them. The calculated volumes were checked by displacement measurements on a few testes which showed the error of the computed value to be small. The texture, color, and vascularity of the gonads were also recorded. The general behavior of the animals was regularly observed, to determine, if possible, any signs of sex activity. A comparison of the animals killed in captivity with those coming directly from the field showed only the usual individual differences.

Technique

For killing and fixing, Zenker's fluid with 8 per cent acetic acid was found to give the best results. All testes were sectioned longitudinally at 6 μ, and only those sections from near the center of the testis were used. This gave representative tissues of uniform thickness throughout the entire work.

For general topography of the testis, presence of spermatozoa, and general interstitial-cell structure, a modification of Flemming's triple stain was developed. With this combination of stains, it was found necessary to mordant with phosphomolybdic acid. The strength of solution, staining time, and sequence were very important. Iron alum hematoxylin was used for the tissues from which nuclear counts were made.

EXPERIMENTAL RESULTS

a. Macroscopic examination of the testis

During the hibernating season, from November to March, the testes were minimal in size, with an average volume of about 40 cu.mm. and a length from 5 to 7 mm. and a diameter from 3 to 4 mm. Both the testis and the epididymis were soft and pliable. The testis color varied from a cream to a deep coral.

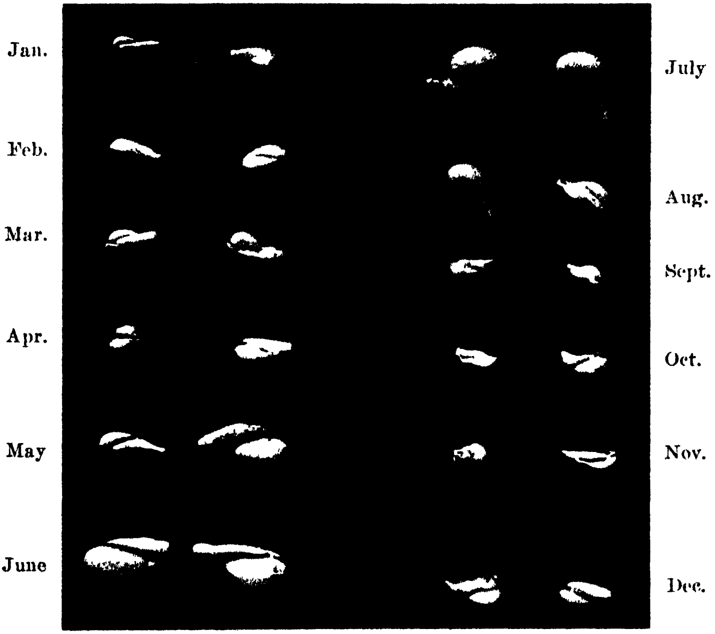

Fig. 1 Seasonal size variations in the horned-toad testis. × $\frac{2}{1}$.

During March, just following hibernation, there was considerable individual variation, because the animals did not all leave hibernation at the same time and those which had been out longer showed greater development. The testes increased very gradually in size during the latter part of March and during April, but in May they enlarged very rapidly (figs. 1 and 2). The seminiferous tubules, as they became larger, stood out prominently under the tunica vaginalis. The testes were turgid and a gray-white in color. The largest testis was taken June 29th and measured 15 mm. in length and 11 mm. in diameter.

During July and August the testes decreased in volume and became less firm in texture. The color was a pink

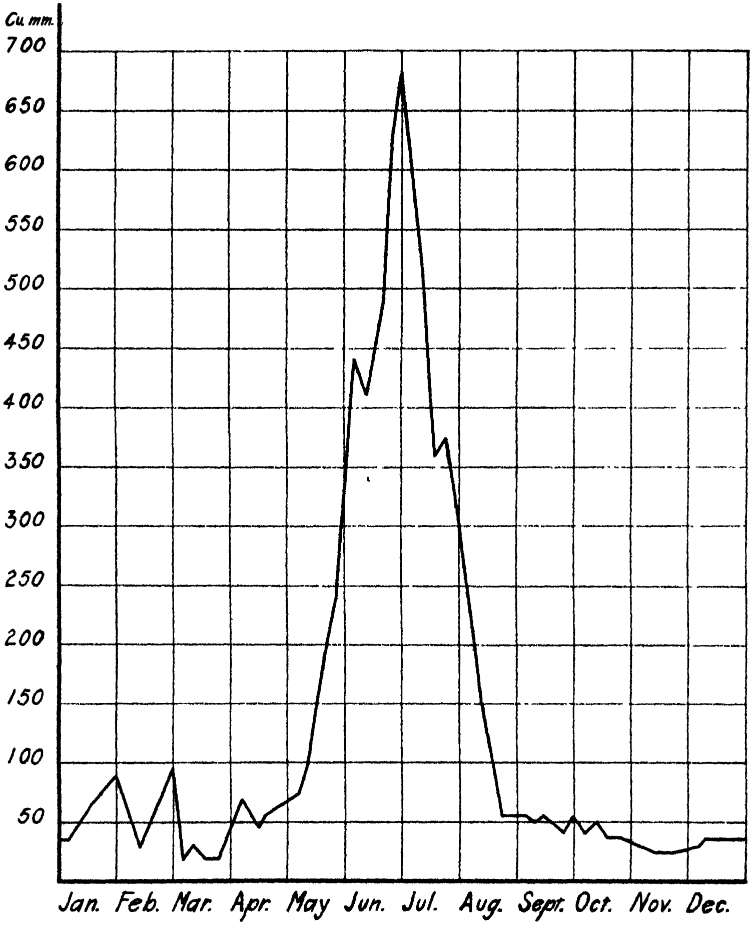

Fig. 2 Graph of testis-volume changes.

or reddish brown and in many cases hemorrhagic in appearance. This was due to the persistence of the increased vascularity characteristic of the breeding season. In the latter part of August the testes were reduced to a very small size and reached the minimum in October.

The maximum average volume was seventeen times the minimum average volume (fig. 2). Volume changes were accompanied by characteristic variations in testicular structure and are correlated with the microscopic findings. The volume variations are in accord with those found in the literature for other animals having a definite breeding season.

b. Macroscopic examination of the ovary

A macroscopic study of the ovary was made in order to correlate its cycle with that of the testis. The ovary underwent seasonal volume variations as did the testis, but with somewhat greater and more sudden changes.

During hibernation the ovaries were small white bodies with the individual follicles 1.5 mm. in diameter. Those with dimensions of 2 mm. × 4 mm. and a volume of about 50 cu.mm. were of minimum size (fig. 3).

During April the activity of the ovaries was shown by a slight increase in the size of the follicles. In May there was a rise in volume, reaching, by the first part of June, a total of about 100 cu.mm. The average follicles stood out prominently and their diameter reached 1.5 mm.

During the latter part of June and the first part of July there was a marked development of the ovaries. By the middle of July they measured about 30 × 25 mm. and the volume reached a maximum of 3600 cu.mm. (fig. 4). The individual follicle diameter was 7 to 10 mm. The developing ova gradually assumed a yellow color and as they matured became a deep orange yellow.

This maximum volume was not maintained for any length of time; ovulation immediately took place. The first ovulation was observed on July 19th and the last on August 10th. The greatest number of ovulations occurred during the week from July 29th to August 5th. That ovulation had taken place was shown by the appearance of the ovaries and by the presence of ova in the oviduct (fig. 3).

By the last of August all signs of ovulation had disappeared, and the condition of hibernation was assumed.

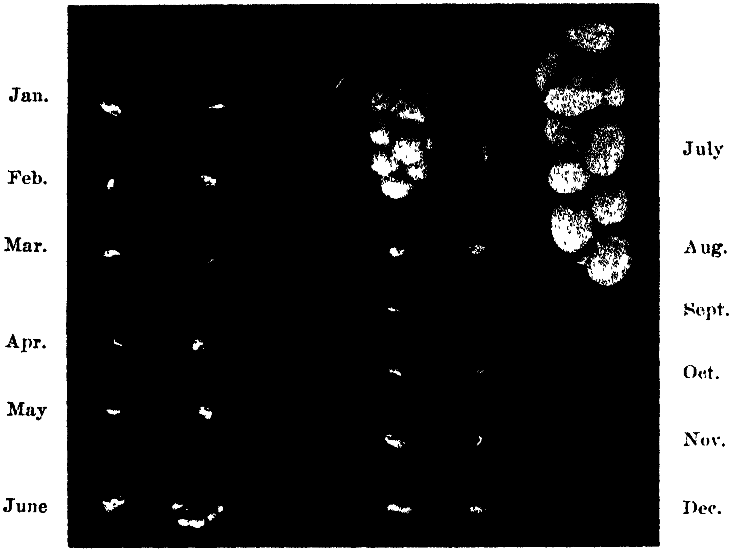

Fig. 3 Seasonal size variations in the horned-toad ovary. × ½. Maximal size is early in July. Ovulation is later in July. The ova from the second ovary, representing July, are in the oviduct and shown to the upper right.

There has been no record of such a series of ovaries noted in the literature. The volume cycle was found to correlate in a definite manner with the testis-volume changes and with the breeding period.

c. General microscopic topography

Microscopic studies showed great contrast between the testes of the hibernation period and those of the breeding season. This was largely due to the relative diameters of the seminiferous tubules and the size of the intertubular spaces.

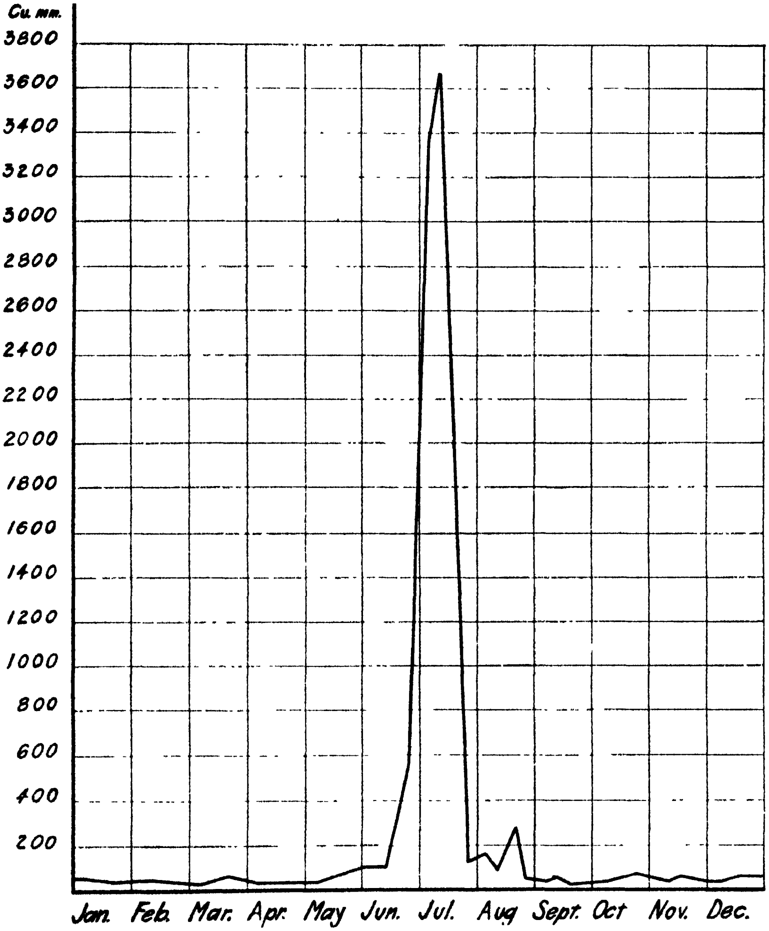

Fig. 4 Graph of ovary-volume changes.

The diameters of the tubules were measured for each month (table 1). These measurements were limited to those tubules which appeared circular in cross-section. Although there were great changes in the tubules at different seasons, in no case was any variation observed in the tubules of different parts of the same testis, except where they join the tubules of the epididymis.

Throughout the hibernation period the tubules were small, with an average diameter of about 100 μ and the basement membrane greatly folded, making the testis a dense compact structure (fig. 6).

As the breeding season approached the tubules began to enlarge, causing the folds of the basement membrane to straighten. This was due to the production of spermatozoa, which were first observed in the testes taken May 16th. The tubules reached a maximum diameter of 364 μ in the latter part of June or the first part of July, when the tubules were gorged with spermatogenetic elements and masses of free sperm were observed in the epididymis. The above condition (fig. 7) was maintained until the latter part of July or the first part of August.

TABLE 1

Seminiferous-tubule diameters and intersection measurements and interstitial-cell numbers

	IN μ	AGE INTER-ION WIDTH IN μ	AVERAGE INTERSECTION LENGTH IN μ	AVERAGE NUMBER OF INTERSTITIAL-CELL NUCLEI PER INTERSECTION
January	95	55	121	30
February	82	44	118	41
March	128	42	97	32
April	118	38	102	43
May	114	32	90	22
June	364	33	80	9
July	306	30	73	10
August:				
First week	226	31	99	13
Second week	170	73	137	22
Third week	95	75	236	72
Fourth week	112	64	139	52
September	108	63	175	68
October	95	73	176	72
November	106	45	131	46
December	96	52	120	45

There was a sudden change at the close of the breeding season, in the middle of August. As the spermatozoa were discharged they left the tubules reduced to mere rings, out-

TABLE 2

Quantitative results

	MONTH							
	April	May	June	July	August, first half	August, second half	September	October
Volume of testis (in cu.mm.)	59	157	598	484	235	61	52	39
Percentage tubules	74	82	92	87	81	54	63	59
Volume of tubules (in cu.mm.)	44	129	553	432	190	33	33	23
Length of tubules (in cm.)	341	1265	532	589	717	389	359	331
Percentage lymph space	1	1	1	4	1	1	2	1
Volume lymph space (in cu.mm.)	0.8	2	7	18	3	0.7	0.9	0.4
Percentage interstitial tissue	32	17	7	9	18	40	36	40
Volume interstitial tissue (in cu.mm.)	19	29	40	42	42	24	19	16
Intersections per unit volume	90	55	23	21	27	61	73	76
Number of interstitial cells per intersection (counted)	43	22	9	10	17	62	68	72
Apparent average increase in interstitial cells per intersection	9	2	—3	0	6	17	17	24
Actual average increase in interstitial cells per intersection	24	10	2	0	1	35	41	38
Ratio of total number of interstitial cells in testis to the number at time of minimum (July)	2.2	1.8	1.2	1	1.1	2.3	2.5	2.1

lined by single layers of cells. Spermatozoa were not found in the testis, with the exception of one individual, after August 12th. In the horned toad the decrease in tubule diameter was largely due to folding of their walls rather than to shrinkage. Perimeter measurements indicated that there was no shrinkage of the basement membrane and that the perimeter remained constant. There has been no reference to this folded condition noted in the literature, although Mazzetti ('11) refers to the tubules of the lizard as having undulated outlines. There was also a great reduction in total tubule length (table 2). These lengths were calculated by the method described by Bascom and Osterud ('25). These changes advanced until the testis had returned to the condition characteristic of hibernation.

d. The interstitial cells

Interstitial cells have certain distinguishing characteristics. They are usually described as polyhedral cells with a round nucleus and abundant cytoplasm, which usually contains inclusions such as pigment granules, crystalloids, or fat globules. The horned-toad testis has the characteristic interstitial cells just described, although, as Mazzetti ('11) found for the lizard he examined, they are much smaller than those found in many other groups of vertebrates.

At the close of the breeding season, in August, the interstitial cells were without definite cell boundaries. The nuclei were round, except when they assumed the shape of the space between fat globules (fig. 9), or when they occurred between tubules and were elongated and flattened so as to appear elliptical. The round nuclei had an average diameter of 4.6 μ. This syncytial condition, characteristic of this period, has been described by Rasmussen ('17) and Humphrey ('21).

As hibernation approached the interstitial cells gradually became definite in outline and polyhedral in form. In December they had dimensions of 8 × 11 μ. The nuclei were round, except where affected by vacuole or tubule pressure, and were 4.6 μ in diameter with a relatively small number of very coarse chromatin granules and very prominent nucleoli.

At the end of hibernation the interstitial cells increased slightly in size, but the nuclear diameter remained constant. In March the cells were 9 × 11 μ and gradually increased until in June they measured 14 × 18 μ (fig. 8). The nucleus increased to 6.8 μ in diameter and was round, with the exception of flattened individuals between the tubules. The maximal size of the interstitial cells in the horned-toad testis accompanied the height of the spermatogenetic cycle. This corresponds to the findings of Rasmussen ('17) for the woodchuck and Humphrey ('21) for the urodele.

e. Changes in the volume of interstitial tissue and the number of interstitial cells

Several workers have based their conclusions upon the variations in the amount of interstitial tissue and its relation to the breeding season without any quantitative work. This has probably led to faulty conclusions, as will be shown later. Bascom ('25) has shown, in his work on the cryptorchid testes of sheep, how the lack of quantitative evaluation of work may lead to errors in interpretation.

In the present study the volume of the interstitial tissue and the volume of seminiferous tissue were calculated and expressed in per cent. To do this, the method described by Bascom ('25) was used, with the exception that volumes, and not weights, of the organs were employed. In addition, the volume of lymph space was calculated. That lymph space should be calculated is indicated by the very large lymph spaces present in certain testes, such as those described by Oslund ('24). Microscopic fields were projected upon tracing-paper and drawn at a magnification of 200 diameters. Each drawing represented a volume the area of the field in extent and 6 μ thick. From these drawings the paper representing the tubules was cut out and weighed, and that representing the interstitial tissue and that representing the lymph space were also weighed. The volume of the lymph space was found to be greatest during the breeding season, probably due to greater vascularity at that time. The recorded weights gave the relation between the volume of the interstitial tissue and the volume of the tubules. By taking a large number of such samples, the constitution of the testis was obtained (table 2). The results show that the volume of interstitial tissue is from 7 to 10 per cent of the total testicular volume during the breeding season and increases to from 30 to 40 per cent in the period that follows. This relative increase in percentage would seem to indicate an actual increase in volume of the interstitial tissue. There is, however, a marked decrease in the total volume of the testis between these two seasons, and if this is taken into consideration, that is, if the volume of the interstitial tissue is calculated from its percentage and from the total testis volume, it will be found that there is an actual decrease in the volume of the interstitial tissue (table 2 and fig. 5). The necessity of making this calculation, in order to prevent error, has been strongly emphasized by Bascom ('25).

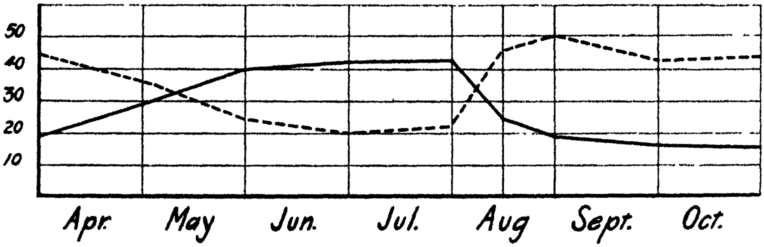

Fig. 5 Graph of interstitial-tissue volume and of interstitial-cell number. Solid line, average interstitial-tissue volume in cubic millimeters. Dotted line, relative interstitial-cell number based upon the number in July. That is, there are 2.5 times as many cells in September as there are in July. See table 2 and text, pages 332, 335.

It has been shown that the volume of interstitial tissue is greatest during the breeding season. If this is true, the conclusions of several workers may be incorrect. It is important, however, to differentiate between volume of tissue and the number of cells.

The number of interstitial cells appears to be greater in August and September than during July. In order to check this carefully, the relative number of interstitial cells for the various periods was determined by making cell counts. Since cell boundaries were indistinct at certain seasons, if not totally lacking, the number of nuclei found was recorded.

As a basis for comparison, the average number of nuclei per intersection was used, that is, the number of nuclei per space between adjoining tubules. A representative group of intersections was obtained, for each testis, by arbitrarily taking those intersections which were arranged upon the vertical axis of the tissue section in question and counting the nuclei in them as they appeared. These counts were made from tissues of uniform thickness, 6 μ, stained with iron alum hematoxylin. This method will, of course, not give an exact quantitative measure of the number of cells, but it serves for an adequate comparative estimate.

During the hibernation season the number of interstitial cells was nearly constant. As the breeding season approached the number per intersection gradually decreased from April with a count of forty-three to July with a count of ten. This minimum was maintained until the third week of August, when the number increased from twelve to over seventy per intersection. This great increase was maintained, except for individual variations, until the latter part of October, when a decline began (table 1). Testes showing these contrasts are pictured in figures 8 and 9. An increase in interstitial-cell numbers at this period has been reported for various animals (Rasmussen, '17; Humphrey, '21; Champy, '24; Aron, '24), but without any actual numerical data.

Correlated with this increase are intersection size variations. In each case the greatest length and an average width of the intersection were taken (table 1). The spaces were so irregular in shape that no accurate values could be obtained, but the figures give an idea of the changes and give a check upon other observations. As an example, these measurements correlate with changes in the number of intersections and with the number of interstitial cells and with the volume of interstitial tissue.

The cell counts alone would indicate that there was an increase of cells after the breeding season. The question arises as to how much of this increase is apparent and how much of it is actual. There is one possible factor which would tend to give apparent increases. If there were a decrease in the number of intersections from one season to the next, there would be an increase in cells per intersection, although the total number in the testis remained constant. The relative number of intersections per testis was therefore determined by counting those appearing in a large number of fields. This gave, for the month, the average number of intersections per unit volume, and by taking into account the volume of the testis, a factor was obtained relative to the number in July. Table 2 gives figures for the following data: 1) Apparent average increases in cell numbers per intersection over the average of July; 2) actual average increases per intersection over that of July; 3) the ratio of total number of cells in the testis for a period to the number for July.

The method of calculation was as follows: Let N_1 be the relative number of unit volumes in the first testis and N_2 be the number of unit volumes in the second testis. Let I_1 be the number of intersections per unit volume in the first and I_2 in the second. Let X_1 be the number of nuclei per intersection in one case and X_2 be the number for the other. If there is no change in the total number of cells in the testis as a whole, between the two periods represented, then $N_1I_1X_1 = N_2I_2X_2$. However, although the total number of cells were constant, the number per intersection would change if the number of intersections changed, and the number of cells per intersection to be expected after such a change may be calculated from the equation by solving for X_2. This change may be spoken of as apparent, since the total number of cells is constant, and the apparent increase or decrease may be calculated by subtracting X_1 from X_2. If the figure obtained for X_2 by calculation does not agree with the figure obtained by counting the cells, then there has been an actual change in the number of cells between the two periods considered. This actual change may be obtained in two ways, either by subtracting (X_2 calculated) from (X_2 counted) or by subtracting the apparent change from the counted increase or decrease.

As an example we may use the months of July and September. Here N_1 is 9.3 and N_2 is 1; the intersections per unit volume are 21 and 73, respectively. The equation then becomes $(9.3)(21)(10) = (1)(73)(X_2)$, and $X_2 = 27$. Without any actual increase in the number of cells between July and September, the number per intersection would be 27, or an apparent increase of 17. But the number counted per intersection was 68. By calculation it was found that the actual increase per intersection, between the two periods, was 41.

To express these changes in terms of the relative number of interstitial cells in the testis as a whole, the following calculations may be made. As before, the equation is $N_1I_1X_1 = N_2I_2X_2$, provided there is no increase in cell numbers between the two periods. But $N_1I_1X_1(\text{July}) = 1953$, and $N_2I_2X_2$ (September) $= 4964$ or $2.54(N_1I_1X_1) = N_2I_2X_2$. Therefore the ratio of the total number of interstitial cells in the testis in September to the number in July is 2.54. These relationships have been calculated for the months from April through October and are recorded in table 2. It is realized that the figures are no more accurate than the method.

There was, then, an actual increase in the number of interstitial cells between the breeding season and the period which followed. There was, however, a decrease in the volume occupied by these cells (fig. 5). From a consideration of the method it will be seen that, in order to compare the amount of interstitial tissue, it is necessary, first, to make cell counts and, secondly, to take into account the volume of the testis. Other workers on seasonal studies have failed to make any numerical estimates of quantity, and thus the results are not reliable.

DISCUSSION

This investigation has shown that in the horned toad interstitial-cell development is closely correlated with the germinal cycle.

The volume changes of the testis were accompanied by an increase in seminiferous-tubule diameter from an average of 114 μ in May to 364 μ in June and by a great increase in total tubule length. This was due to the production of spermatozoa, and the increase of the testis volume began at the time of first spermatozoa production (fig. 2). The ovarian changes were exactly correlated with the changes in the testis.

The height of the breeding season occurred early in July. Copulation was observed in the laboratory on July 7th, and July was also the time of ovulation.

As the spermatozoa were discharged the seminiferous tubules decreased from about 360 μ to about 100 μ in diameter, and the total tubule length decreased from about 700 cm. to about 350 cm. The testis decreased in volume as a result. Although these changes were accompanied by an increase in the individual intersection space, there was a decrease in the actual volume of interstitial tissue. There was, however, an increase in the number of interstitial cells (fig. 5).

After this period, the testes gradually assumed the condition characteristic of hibernation.

Some workers claim that the interstitial cells revert to a connective-tissue-cell type as the breeding season approaches, which is the only type found during this period. They have been described as connective-tissue cells with elongated, flattened, elliptical nuclei, which, following the breeding season, fill out and become typical interstitial cells (Mazzetti, '11; Rasmussen, '17; Humphrey, '21). Mazzetti ('11) says that the interstitial cells in the frog develop from connective tissue and can be observed in all the forms through which they pass. In the present investigation there has been no indication of such a reversion of interstitial cells to connective-tissue-cell type. There are flattened nuclei between the tubules, but they are only the interstitial-cell nuclei flattened by pressure.

It must be emphasized that there are at all times interstitial cells in the center of the intersection where the pressure is less, and that at no time are these flattened cells, between the tubules, absent.

There is disagreement among workers as to the time when interstitial cells are most numerous. Although Marshall ('11) and others have found the increase in numbers to occur during the breeding season, the majority have found that they increase after the breeding season is over (Tandler and Gross, '11; Humphrey, '21; Champy, '24; Aron, '24 b). None of these workers has given numerical data, but has judged only from the appearance of the preparations. That there is really an increase in interstitial tissue has been questioned. Is this increase in numbers real or is it only apparent? Moore ('26) questions this hypertrophy on the basis that it has no constant relation to the degeneration of the tubules and that the decrease in testis volume would naturally restrict the nuclei present to smaller areas and thus cause them to appear more numerous in relation to the tubules. Oslund ('24) states that there is not, necessarily, hypertrophy of interstitial tissue when the tubules are caused to degenerate in experimental animals. If this lack of constant relationships exists in experimental animals, should it be expected in normal ones? Possible error in cell numbers due to apparent increases has been very carefully checked in the present investigation. It has been shown necessary to take into account not only the relative number of interstitial cells, but also the volume of the testis. It is certain that there is an increase in the number of nuclei per testis just following the close of the breeding season, although no mitotic figures were observed. Mitotic figures seem to be extremely difficult to find in interstitial tissue. Humphrey ('21) reports that but few were found. Neither Mazzetti ('11) nor Rasmussen ('17) found any, and Moore ('24 a) states that such division figures are extremely rare.

Several workers have concluded that the interstitial cells have no function in the production of hormones. This conclusion has been based upon the fact that interstitial tissue was thought to be at a minimum during the breeding season and to increase in the period which followed. For this reason the relationships here reported are important.

The quantitative data of the present work show that the time of greatest interstitial-cell numbers does follow the breeding season. However, the time of greatest interstitial-tissue volume is during the breeding season (fig. 5). Which is more important, volume of tissue or number of cells, as far as hormone production is concerned? The activity of interstitial tissue will be discussed in a later paper.

SUMMARY AND CONCLUSIONS

1. There is a definite interstitial-cell cycle in the testis of the horned toad, Phrynosoma solare.

2. To determine the relative amounts of interstitial tissue at different seasons, it is necessary to take into account the following factors:

a. The percentage of interstitial tissue (with lymph space eliminated).

b. The total volume of the testis.

c. The number of cells per unit of volume.

d. The relative total number of interstitial cells.

3. The diameter and the total length of the seminiferous tubules vary with the seasons. The maximum is reached during the breeding season.

4. The interstitial cells of the horned toad do not revert to a connective-tissue-cell type for—

a. The flattened nuclei found between the seminiferous tubules at times are those of interstitial cells flattened by pressure.

b. These flattened nuclei do not occur in the center of the intersections where there is less pressure.

c. There is no time when typical interstitial cells are not present in the testis.

d. There are at all times some flattened interstitial-cell nuclei between the tubules, but these have no definite cycle.

5. There is an exact correlation between the ovarian-volume cycle and the time of ovulation with the testicular cycles.

6. The interstitial-cell cycle is definitely correlated with the spermatogenetic cycle.

a. The greatest size of the interstitial cells is correlated with the height of the breeding season.

b. The interstitial-cell number decreases as the breeding season advances and as the spermatozoa are produced. The minimum number is reached at the height of the breeding season.

c. The increase in interstitial-cell numbers comes when the spermatozoa are extruded and the breeding season is over.

d. The volume of interstitial tissue is greatest during the breeding season and decreases at its close.

7. If the interstitial tissue is responsible for sex characters and activity, the results of the present work would indicate that it is the volume of tissue, and not the number of cells, that is important.

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PLATE 1

EXPLANATION OF FIGURES

6 General topography of testis during December. × 125. The tubules are of small diameter, with folded basement membrane and without lumen. Spermatogenesis is absent. There is, relatively, a large amount of interstitial tissue. (See text description, p. 328.)

7 General topography of testis during July. × 125. The tubules are of extreme size, with spermatogenesis at its height. There is only a small amount of interstitial tissue present. (Compare text, p. 328.)

8 Tubular intersection typical of testis during July. × 700. At this time the number of interstitial cells per intersection is minimal. However, the cells and also their nuclei are of maximal size. (Compare text, pp. 331, 333.)

9 Tubular intersection typical of testes in late August. × 400. There is, following the breeding season, a relative abundance of interstitial tissue in the individual intersections. The tissue is syncytial in character, but there are a large number of nuclei. These nuclei are relatively small and may be flattened by the pressure of large fat globules present in the cytoplasm. (Compare text, pp. 330, 333.)

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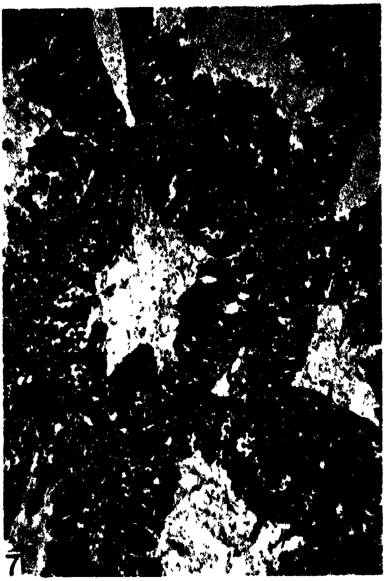
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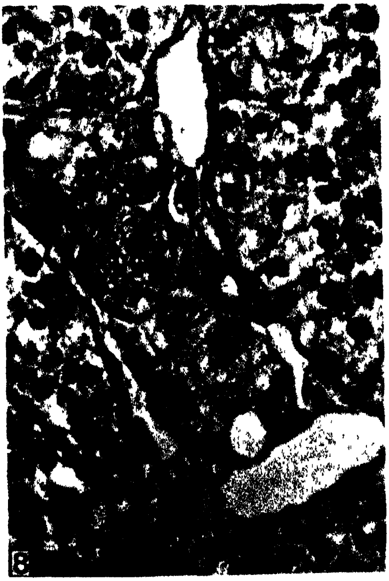
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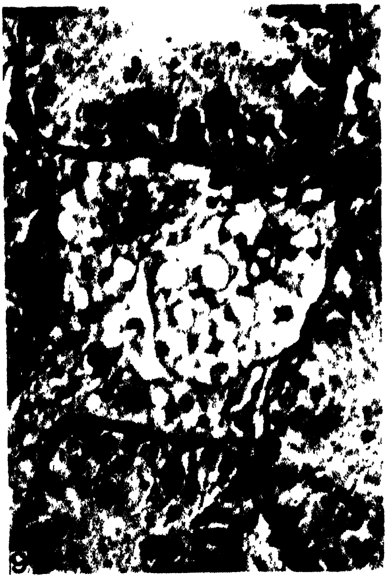
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INTERSEXUALITY IN RANA CANTABRIGENSIS [1]

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FIVE FIGURES

AUTHOR'S ABSTRACT

The specimen described presents a typical intersexual condition, in which not only the primary sex characters, but also the accessory sex organs and the secondary sex characters are of a mixed type. Both gonads are ovotestes, in which the ovarian as well as the testicular tissues are morphologically normal, but functionally underdeveloped Stromal hyperplasia is distinct, especially so in the left gonad, it is probably a functional factor in the generative process of the spermatic tissue.

Sex transformation in the intersexual frogs may probably be initiated by an abnormality in sex differentiation or in the genetical constitution of sex, and is only subsequently influenced and modified by environmental action Absolute dominancy of male sex in the process of sex transformation is questionable There are indications that in frogs sex transformation may possibly occur in a female direction.

Sex-organ anomaly in anurans was first recorded by Spengel in 1876. Since then numerous cases have been recorded, most of which have been classed as abnormal hermaphrodites. These hermaphrodites may be regarded as intersexual forms, the generative systems of which come to possess elements of both sexes, probably through a genetical abnormality in the sex constitution of the zygote, or through some defective embryonic physiological mechanism inducing a certain specific derangement during sex differentiation, or through environmental influences, as advocated by many, or through any combination of the above factors. Crew ('21 b), in his review of the recorded cases of abnormality of the reproductive system in frogs and toads, enumerated forty cases. Swingle ('22) added another case described in the bullfrog (apparently by Clemens, '21), making a total of forty-one. This number, in consequence, has frequently been referred to as a complete record of known cases up to 1921. A search of literature, however, reveals numerous cases which both of the above-mentioned writers have apparently overlooked. Balbiani ('79), Hoffmann ('86), Beard (Cole, '96),[2] Friedmann ('98 a), and Champy ('13) reported the occurrence of ovarian elements in the testes of anurans; Pflüger ('82), Meyer (Spengel, '84),[3] Eismond ('92),[4] Friedmann ('98 b), King ('10), Swingle ('17), and Witschi ('14, '21) observed the same phenomenon among frogs. Matthews ('85) noted the presence of 'ovaries' and more or less perfect müllerian ducts in a great number of male toads. Ikeda ('96) recorded hermaphroditism in the Japanese toads, Goodall ('08), in Rana temporaria, and Yung ('07), in Rana esculenta, in which species Kuschakewitsch ('11) also found a lateral hermaphrodite. Loisel ('01) described a female frog of Rana temporaria which possessed very degenerate ovaries and male secondary sex characters. Nussbaum ('06, '12) noticed in Rana fusca abnormal conditions of testes in forms of hypertrophy, atrophy, and multiplicity. Of the last phenomenon, Gerhartz ('06) also recorded two specimens. Fuhrmann ('13) presented forty interesting cases of hermaphrodite toads, ranging from 'hermaphrodisme rudimentaire' to 'hermaphrodisme effectif.' Heymons ('17) reported a male frog (Rana temporaria) in which the right gonad was a hypertrophied testis, while the left one was filled with dark brown globules, müllerian ducts of the female type being present. Patzelt ('18) described an adult female of Rana esculenta with rudimentary ovaries and small oviducts; Bhattacharya and Das ('20), an adult male of Rana tigrina with testicular atrophy and imperfectly developed müllerian ducts; Lloyd ('21), a male frog of Rana temporaria possessing only one greatly enlarged testis. Takahashi, in 1915, found two cases of intersexuality in Bufo bufo japonicus and, in 1917, five more in the same species and, besides, one case in Rana limnocharis. According to Harms ('21 a), "In der Umgebung von Marburg findet man nicht selten äusserlich männlich aussehende Kröten . . . ., die zwischen Hoden und Bidderschem Organ entweder einseitig oder beiderseitig ein mehr oder weniger grosses Ovarium besitzen." Harms ('21 b) also reported castration experiments on some glandular hermaphrodites of toads. In view of the above brief résumé of the more important literature, it becomes evident that Crew-Swingle's list of forty-one cases is by no means complete; on the contrary, it represents but a fraction of the large number of cases known up to that time.

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[1] Contribution from the Zoölogical Laboratory of the University of Michigan.

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[2] Cole mentioned that Doctor Beard had observed well-developed ova in the testis of the toad quite apart from Bidder's organ.

[3] According to Spengel, Fr. Meyer had informed him, "dass nach seinen Erfahrungen sehr häufig bei Fröschen Hoden mit Ovarialeinschlüssen und umgekehrt Ovarien mit Hodeneinschlüssen vorkämen."

[4] Data from Mitrophanow ('94), Cole ('96), and Ognew ('06).

Since 1921, more cases of abnormal sex organs have been described in frogs and toads. Cunningham and Becton ('26), Caroli ('26 a, b; '27), and Stohler ('28) found female elements in the male gonads of Bufo. The last-named author also claimed that he had found a 'genetischen Hermaphrodit' possessing the functional as well as potential units of both sexes. Guyénot and Ponse ('23 b) recorded two hermaphrodite toads from which they had removed the testes. Harms ('26) found 1 per cent of intersexuality among male toads in Königsberg. Eggert ('26, '27, '29) reported many cases of intersexuality in Bufo vulgaris, and ('29) one case in Rana temporaria. Caroli ('26 c) observed, in the left kidney of a sexually mature male toad, a globular formation composed of spermatozoa, spermatocytes, and spermatogonia. Van Oordt ('22) found in Rana fusca numerous eggs in the right testis of an otherwise normal male. Witschi ('21, '23) described cases of hermaphrodites which he regarded as masculinized females. The same author ('24) discussed some gonadic anomalies, the cause of which he attributed to the influence of delayed fertilization of the eggs concerned. Crew and Fell ('22) found in a male of Rana temporaria a displaced testis which contained many large bodies closely resembling ova. According to the authors, these ovum-like bodies were not ovarian in nature, but were merely a product of liquefaction of the degenerating spermatozoa. Rau and Gatenby ('23) described a partially hermaphroditic frog and mentioned in passing that in Bufo some rare instances of hermaphroditism had been observed during their study. In Rana esculenta, Patzelt ('23) noted two cases of testicular atrophy; Dornesco ('26, '27), two of congenital monorchidism, and Dubois et Ponse ('27), five of hypogenitalism. In Rana temporaria, Dauvart ('26, '27) recorded two specimens of heterotopic testis, and Shaw and Brambell ('28), one of aberrant ovary. In Rana pipiens, Maclean ('28) reported a male frog possessing müllerian ducts of female type. Chidester ('26) stated that in two adult male bullfrogs normal eggs were discovered embedded in the wall of the digestive tubes, attached to the fat-bodies and enmeshed in the peritoneum and mesentery. In Rana catesbiana, Swingle ('26) found oocyte-like cells in adult testes and described some larval gonads which were "neither truly female or male, but strongly intersexual, with female characters predominating." Other cases of abnormal frogs with ovotesticular gonads were recorded by Leigh-Sharpe ('22) (species not mentioned), Neal[5] ('24) in Rana pipiens, and Woronzowa ('26), Draigoiu et Pop ('27), and Lloyd ('29), in Rana temporaria. Of all the forms hitherto described, only a few belong to the American species. In this paper is presented the first record of intersexuality in the American wood frog, Rana cantabrigensis.

DESCRIPTION

The animal to be described was caught, together with a number of other wood frogs, in the afternoon of September 29, 1928, in a pond of White Woods in the vicinity of Ann Arbor, Michigan. It was killed the following evening in Bouin's fixing fluid and preserved in 75 per cent alcohol, where it remained until recently, when the writer began examining and sectioning the frogs in his study of the seasonal variations of the gonads. The intersexual nature of the frog first manifested itself in the gonads, which in size were testicular, while in texture distinctly ovarian, except for the posterior portion of the left gonad. Upon closer examination, rudimentary müllerian ducts were found present along the outer border of both kidneys. From these preliminary findings, the author deemed it worth while to make a detailed study, the results of which are embodied in the following description.

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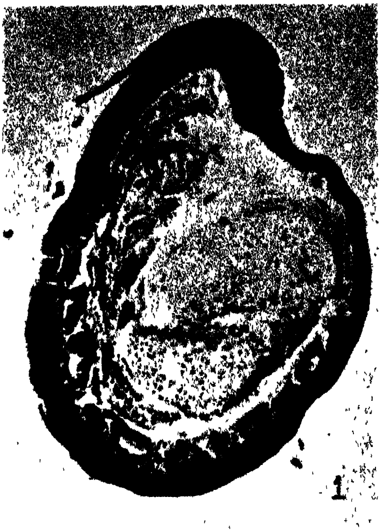
[5] The author spoke of a large Bidder's organ associated with the left testis. What he meant by Bidder's organ might be an abnormal ovary. It is now generally agreed that Bidder's organ as such does not occur in Rana. According to Müller ('30), a rounded body was found present at the anterior end of the testes in frog tadpoles. His observation has not been confirmed.

External characters

The specimen was a full-sized adult frog. It measured 36 mm. from the tip of the snout to the posterior surface of the ischial symphysis. Measurements of scores of wood frogs killed at different times of the year showed that the average body length in males was approximately 38 mm. and in females, nearly 42 mm. The difference, though considerable, cannot, however, be considered significant for identifying sex in any particular case, since males and females frequently merge into the length range of the opposite sex. In color, the frog was grayish, with a darker color along the midline of the back and with black bars and spots on both hind limbs. Such coloration is characteristic of many females, though also present in some males; but it is decidedly different from the extreme dark condition so typical of many males at this season. In body build the frog resembled more a small female than a male of about the same age. The skin of the back and along the upper surface of the hind limbs was roughened with many distinct papillae having the appearance of small, rounded, pale grayish or white, warty growths. These papillae were most dense around the anal region, becoming more and more scattered anteriorly until totally absent at some distance behind the eyes. Along the sides of the trunk a few larger elevations were noticed. The distribution of the papillae as a whole suggested femaleness. In males such papillae, as a rule, are absent, and, if present, are usually scanty.



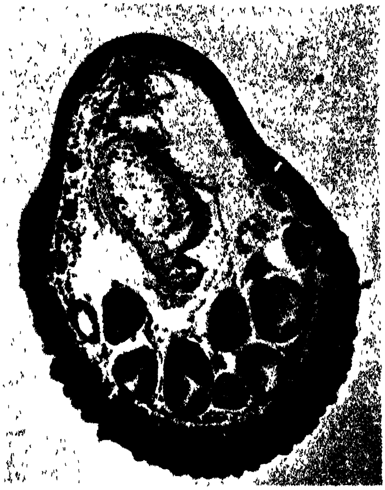


Fig. 1 A transverse section of the supplemental toe (second finger) of the intersexual frog. Note absence of epidermal papillae and the rudimentary condition of mucous glands.

Fig. 2 A transverse section of the supplemental toe of a normal male frog (killed October 10th), showing well-developed epidermal papillae and mucous glands.

On the base of the mani there were definite swellings, in form similar to, in size, however, a little smaller than the finger pads of normal males. They were also pigmented in the usual male pattern, though only faintly so. Histologically, however, they were found to lack all projecting epidermal structures and their mucous glands were few and small (fig. 1). With respect to these two features, they showed a strong contrast to the condition so well displayed in most healthy males of this season (fig. 2). The webs of the hind feet are in most cases distinctly different in the two sexes: they are concave in females, whereas in males they may be convex or less concave than in the female. In our intersexual specimen the webs were nearly flat-margined, representing in a way an intermediate condition between the extreme concavity in the female and convexity in the male.

Accessory sex organs

1. Male. The wolffian ducts originated on the dorsal surface of the kidney and became gradually shifted to its outer border, very close to the renal portal vein. Histologically, the ducts were lined with a columnar epithelium, which was in some places distinctly folded. These wolffian ducts were exceptionally small anteriorly, as compared with those of normal males; but they grew larger in caliber posteriorly. Below the kidneys they were swollen to form the usual seminal vesicles, both of which measured about 2 mm. in length and were typically male in all respects. Behind the seminal vesicles the ducts ran inward, terminating by two separate openings into the cloaca.

Vasa efferentia were present on both sides. On the left side their number was not observed. On the right side there were ten vasa efferentia distributed over the entire length of the gonad. These did not branch much, only two or three of them bifurcating at acute angles just before entering the kidney.

2. Female. There were two rudimentary müllerian ducts extending from the summit of the seminal vesicles almost to the roots of the lungs, where the oviducts in females normally terminate. In length the right duct measured around 20 mm., being longer than the left, which measured nearly 15 mm. Both of these ducts were thin and delicate, becoming even more so as they proceeded forward. Both ran, for the middle one-third of their course, lateral to the kidneys, with which they were intimately associated by membranous connections and numerous fine cords. Behind the kidneys they were closely bound together with the wolffian ducts and appeared to merge into the seminal vesicles, and finally lost their external identity.

Neither one of the müllerian ducts possessed any dilatation and both were devoid of the characteristic convolutions of normal oviducts, only the right müllerian duct coiling for a short distance above the level of the kidneys. In the posterior two-thirds of the ducts, along their outer side, there were seen small pigmented masses of various shapes, the significance of which was not determined.

Macroscopically, the müllerian ducts showed no anterior opening into the body cavity. Subsequent microscopical examination of cross-sections of the ducts not only confirmed this, but further demonstrated that both ducts were mere solid cords in the anterior third of their course. Posteriorly, they possessed a tiny lumen, surrounded by a single layer of cuboidal cells, and were entirely devoid of any glandular structures. As mentioned previously, the left müllerian duct was shorter than the right; its lumen was also found to be smaller.

Although externally the müllerian ducts appeared to have fused with the seminal vesicles of the respective sides, yet sections of both wolffian ducts and müllerian ducts revealed that their lumina, although closely associated, were distinct and that in no place did they show any indication of fusion. They opened separately into the cloaca. The opening of the left müllerian duct was about 10 μ anterior to that of the right one. About 0.12 mm. posterior to the latter opening were found the orifices of the wolffian ducts.

Primary sex characters

In size the left gonad was larger than the right one. In length the former measured 4.8 mm. and the latter, about 4.0 mm.; widths and thicknesses were too irregular to allow measurements for comparison. In form the two gonads resembled neither testes nor ovaries. The left gonad was somewhat pear-shaped with ovum-like projections around its anterior pole and with numerous minute convexities in its larger posterior portion. These outside convexities were later found to correspond to the spermatic lobules inside. The right gonad was irregular in outline, with constrictions here and there, and showed protruding ova throughout its entire surface. The two gonads were not situated at the same level, the left one being a little behind the right. Both gonads were entirely free from pigmentation.

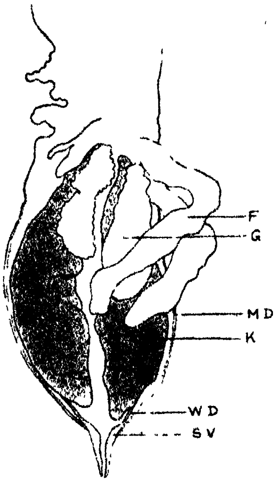


Fig. 3 A semidiagrammatic ventral view of the urogenital system, including parts of both fat bodies. *F*, fat-body; *G*, gonad; *MD*, mullerian duct; *K*, kidney; *WD*, wolffian duct; *SV*, seminal vesicle. Figure outlined with the aid of a camera lucida. Magnification, approximately × 4.

With respect to the corpora adiposa, neither of them presented any unusual features. The right one was compact with but few lobes, while the left one was much more massive, with several big divergent branches, some of which turned backward and capped over the left gonad.

Upon sectioning, both gonads proved to be ovotestes. Their microscopical structures were as follows:

1. The left gonad was found to be composed of a larger testis portion, with a smaller ovary portion situated anteriorly. These two portions were not discrete and the line of junction between them was very irregular. Around the junctional region the stromal tissue was evidently hypertrophied. This hypertrophic stroma appeared to have penetrated for some distance into the substance of the testis portion along a longitudinal, nearly central axis of the gonad, and became gradually thinned out in divergent intertubular branches. Embedded in and throughout the main core of the stromal hyperplastic substance there were found irregular lobules of varying forms and sizes, containing male germ cells mostly in gonial stages. In one instance several tiny bunches of spermatozoa were observed, enclosed in a small space in the overgrown stromal tissue, and tightly surrounded by a heavy mesothelial investment. These phenomena might be interpreted as evidences of degeneration of the spermatic tissue by excessive growth and consequent sclerosis of its interstitium, followed by a deformity and degeneration of the spermatic tubules involved. But a detailed study of the entire gonad showed clearly that in the most posterior portion of the gonad the testicular tubules were well developed with spermatogonia and numerous spermatocytes. In its middle portion, where the stromal hyperplasia was most pronounced, there were found around the main core of the hyperplastic stroma many spermatic tubules in various degrees of formation. In these tubules spermatogonia and occasionally spermatocytes were in evidence. The most anterior portion of the gonad was essentially ovarian, but the stromal mesenchyme appeared to have invaded it from the testicular por-

tion. In the stromal mesenchyme scattered follicles of male germ cells were detected. Their presence here authenticated an invasion of the testicular substance into the ovarian portion. The topography of the whole gonad seemed, thus, to indicate that the hyperplastic stromal tissue was withdrawn from the region where definitive spermatic tubules had been formed and was concentrating in those places where such tubules were being formed or were probably to be formed. This being the case, the conclusion seemed justified that the stromal hyperplasia was probably not a feature of degeneration, but was apparently functional in the organization of testicular structures by serving as a ground-substance in which the various elements of the germinal and somatic tissues aggregated themselves to form the spermatic tubules.

In this connection it should be mentioned that the stroma cells in the hypertrophied condition were structurally normal in all respects. They were without distinct cell boundaries, but each one of them possessed a well-defined nucleus. Their nuclei were always small, compact, and more or less elongated, and contained one, or occasionally two or three nucleoli and irregular strands of shadowy outline. In sharp contrast to this type, the spermatogonial nuclei were large, round or polymorphic in appearance, stained lightly, and contained, in most cases, an intricate network upon which were scattered here and there several distinct nucleoli, usually spherical and of large size.

Careful study of the male germ cells revealed nothing morphologically abnormal. Spermatogenesis was in progress and mitosis normal in every respect. However, except for a few small groups in abnormal position, spermatozoa were not found throughout the entire gland. In sections of normal testes of this season, well-developed spermatozoa were found abundant in the lumina of the seminiferous tubules.

In the ovary portion ova were numerous. But they were not all confined to this portion. Some were found among the testicular structures and even within the spermatic tubules. Such ova were morphologically identical with those in the ovary portion of the gonad, and being so entirely different from their neighboring male sex cells, with which they were not connected by any intermediate forms, naturally could not be regarded, at least from morphological evidence, as the products of the male germinal tissue. Rather they should be considered the remains of previously existing ovarian tissue which had been replaced by spermatic tubules. The presence of an ovum in a seminal tubule did not result in any recognizable deformity of the male germ cells found therein, but the shape of the ovum seemed to be conditioned by the form of the tubule in which it was contained.

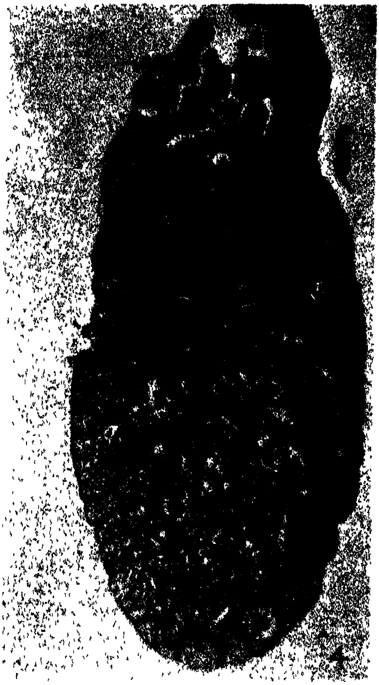


Fig. 4 A horizontal section of the left gonad, showing its ovotesticular structure. Note stromal hyperplasia in the middle region of the gonad, and testis-ova in different parts of the testis portion. Sections were cut at 8 μ and stained with iron hematoxylin.

Measurements of ova showed that their diameters varied from 100 µ to about 250 µ, with the mean around 200 µ. All ova, when carefully examined, were found to be surrounded by a delicate capsule of flattened follicle cells which were stretched to an extreme thinness. The cytoplasm of the ova was homogeneous and stained grayish with iron hematoxylin.

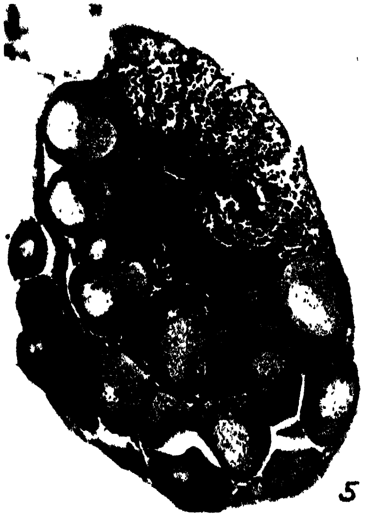


Fig. 5 A transverse section through the posterior part of the right gonad. Testicular structures are distinct in the dorsomedial portion of the gonad. Many primordial germ cells either singly or in lobules, which also contain spermatogonia, are found distributed in the hypertrophied stroma. Sections were cut at 10 µ and stained with iron hematoxylin.

The nucleus stained only lightly and was generally round or oval with a sharply defined nuclear membrane. In the nucleus there were many intensely basophilic bodies of various sizes, more abundant toward the periphery than in the central portion. An intranuclear network of irregular, plumous filaments was quite distinct in some cases. The ova did not show any sign of true yolk formation. Apparently, owing to certain unfavorable conditions, they were prevented from undergoing functional development. In normal ova of this season yolk was formed in abundance, increasing the size of the ova to a diameter of over 1000 μ.

2. In the right ovotestis the germinal tissue was everywhere ovarian, except for a small patch of spermatic substance. In the anterior portion of the gonad the interstitium was very scanty and loose, and the ova appeared to be 'floating' in the places they occupied. Posteriorly, the stromal tissue became distinctly hypertrophied in some places. In close association with the hyperplastic stroma one found many spermatic tubules, most of which were grouped along the dorsomedial border of the gonad. Similar to the condition in the left ovotestis, the excessive growth of the stromal tissue in this ovotestis was apparently not a sign of degeneracy, but was best regarded as associated with the generative process of the testicular structures. Histologically, the spermatic tubules were normal, only of small size, and the germ cells contained in them were mostly in primordial and gonial stages, though occasionally spermatocytes were found, but no spermatozoa. These conditions indicated that the seminal tubules were newly formed. Here and there in the hypertrophied stroma some tubules were seen still in the process of formation. The ova so abundant in this gonad were similar in structure to those in the left gonad. The follicular walls were distinct and intact, closely adhering to the cell surface. Pigmentation was absent.

Summary of the important sex characters

Male characteristics	*Female characteristics*
Presence of finger pads	Abundance of dermal papillae
Web condition of the hind limbs male-like	Light body coloration
Vasa efferentia on both sides	Two rudimentary müllerian ducts
Two seminal vesicles	Separate openings of the müllerian ducts and the wolffian ducts into the cloaca
Large testis portion in the left ovotestis	Small ovary portion in the left ovotestis and many ova in the testis portion of the same gonad
Small patch of spermatic tissue in the right ovotestis	Large ovary portion in the right ovotestis

DISCUSSION

Secondary sex characters

From a superficial examination of the secondary sex characters, the above frog would be classed as a male, due to the presence of well-defined finger pads. However, sections of the pads show distinctly that they lack the characteristic papillae and glands found in the normal males. Careful inspection of other external features reveals also that the animal is not a normal male, but of an intersexual type.

A study of literature on genital abnormalities in frogs shows that most of the earlier writers on this subject neglected to describe carefully the external sex characters. In many accounts the authors merely mentioned the 'sex' of their specimens, based, in all probability, on a superficial examination of the presence or absence of the finger pads. Such diagnosis of sex is not adequate, as is shown in the present case.

Concerning the relationship between primary and secondary sex characters, it is noted that in our frog, despite the asymmetrical condition of the gonadic constituents, the secondary sex characters are developed in an almost perfectly symmetrical fashion. There is, thus, no side-to-side correlation between the primary sex characters and the secondary sex characters. But the intersexual condition of the latter is certainly correlated with the mixed constitution of the former.

Accessory sex organs

In our specimen vasa efferentia and seminal vesicles are both present. On the right side, vasa efferentia are found not only in connection with the testis portion, but also with the ovary portion of the ovotestis. Besides these male accessory sex organs, there are two rudimentary müllerian ducts, the left duct being the less developed. This asymmetry corresponds in a rough way to the relative amount of ovarian tissue in the respective gonads. Positive correlation between the degree of development of the müllerian ducts and the amount of the ovarian tissue in the gonads has been both affirmed and contradicted in specimens of intersexual frogs. In toads Sutton ('85) examined more than 250 specimens and found "as a rule, the amount of development of these Müllerian ducts is in direct proportion to the size of 'Bidder's organ'."[6] Fuhrmann, from his extensive study of hermaphrodite toads, came to the conclusion that there was no correlation whatsoever between the development of the müllerian ducts and the condition of the ovaries in the hermaphrodites. Crew maintained that "the degree of development of the Müllerian duct is controlled to some extent by the activity of the ovary of the same side," and that in the process of sex reversal, "the appearance of the spermatic tissue is associated with the cessation of the activities of the ovaries and the further development of the Müllerian ducts is thereby prohibited. The degree of development of these ducts is some indication, therefore, of the relative time when the transformation of the gonads began." Witschi ('21) concluded from his observation on asymmetrical hermaphrodites that "somatische Geschlechtsmerkmale von den Keimdrüsen abhängig sein können, ohne dass eine innere Sekretion mitwirkt." In 1925, he further demonstrated that not only the müllerian ducts, but also the vasa efferentia and seminal vesicles showed "eine einseitige Korrelation zur Keimdrüse." With respect to our specimen, however, the seminal vesicles are similar on both sides, notwithstanding the differing amount and condition of the testicular tissue in the two gonads.

Primary sex characters

In literature the presence of two intersexual gonads in one frog has been recorded in many cases. In Rana temporaria, Marshall ('84), Kent (Crew)[7] ('85), Latter ('90), Cole ('96),

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[6] By 'Bidder's organ' the author referred to the rudimentary ovary or potential ovary in the normal toad, and not to the abnormal ovary as is found in the hermaphrodite toad.

[7] Kent's specimen was redescribed by Crew in 1921.

Punnett ('00), Crew ('21 a), Witschi ('23), and Woronzowa ('26) reported cases where both gonads were microscopically demonstrated to be either ovotestes or ovum-containing testes.[8] Ridewood ('88), Smith ('90), and Lloyd ('29) had specimens also of Rana temporaria, in which, upon external inspection, two ovotestes were present. Of these, however, no sections were made. In Rana esculenta, Crew described a specimen with two ovotestes, and Mitrophanow ('94) a frog, in which there were found two testes of unequal size, both containing doubtful ova; the right one, however, containing one true ovum and an anterior lobe which was regarded as a rudimentary Bidder's organ. In Rana viridis, Friedmann ('98 a) observed female elements in both testes of an adult specimen. In Rana fusca, Hooker ('12) and Boulangé ('14) found two ovotestes in intersexual individuals. In American species, Swingle ('17) mentioned that in a pseudohermaphrodite of Rana pipiens, both testes contained ripe spermatozoa and large oocytes. Clemens ('21) dissected a bullfrog which contained an ovotestis on the left side, while the right gonad had been destroyed before the hermaphroditic nature was discovered.

In most of the above-mentioned cases the ovarian tissues were regarded as pathological or degenerating, while the testicular tissues were considered normal. In our specimen, from a comparative study of the histological and cytological structures in the ovotestes and in normal gonads of the same season, it is shown that both ovarian and testicular tissues of the ovotestes, though apparently morphologically normal, are functionally underdeveloped. The spermatic tissue has failed to generate mature spermatozoa, while the ova show no sign whatsoever of true vitellogenesis. It has also been shown that the interstitial hyperplasia is not necessarily an actual feature of tissue degeneration, as so recorded in many cases, but is probably a factor in the formation of testicular structures during their replacement of the ovarian tissue. If this is the case, we may interpret this frog as undergoing a sexual transformation toward maleness.

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[8] An ovotestis is composed of recognizable portions of ovary and testis, while an ovum-containing testis is mainly testicular in structure, containing an ovum, ova, or groups of ova in its spermatic substance.

General considerations

Crew, in his attempt to explain a female-to-male sex reversal in frogs, has strongly contended that the spermatic tissue is dominant over the ovarian tissue and that the appearance of a small amount of the former is associated with the immediate degeneration of the latter. "Quantity, apparently, is not a factor, for a very small nodule of testicular substance can still provide sufficient of the male hormone to wipe out the great mass of ovarian tissue and its abundant internal secretion." In our frog the presence of two small müllerian ducts may be taken, in Crew's interpretation, as an indication that sex transformation toward maleness has become active during, if not before, the time of metamorphosis. Judging from the size and body build of the frog, it is at least three years from metamorphosis. If the process of sex transformation has been going on for three or four years, one would naturally expect, in accordance with Crew's notion of the dominancy of the male substance, a fully transformed individual with typical testes. But, on the contrary, we find two gonads still of ovotesticular structure and also that in the right gonad the male element occupies but a small portion, while the female element strongly predominates in quantity. Evidently, this case does not support Crew's contention of the absolute dominance of the male substance and indicates that the strength of both sex substances is conditioned by the environmental complex. Under certain environmental conditions, one sex may be favored in development, while the other tends to degenerate. Under certain other environmental conditions, it is conceivable that both sexes can suitably exist together for some time. Eventually, however, one sex or the other will gradually fade out of existence, depending upon which sex is favored in development, and not upon the alleged dominancy of any particular sex.

The breeding experiments of Crew and Witschi on some hermaphrodite frogs seemed to show that those frogs were genetically females undergoing masculation. In our case the presence of finger pads and of well-developed vasa efferentia and seminal vesicles, as well as the prevailing tendency toward maleness in the gonads, may also be regarded as morphological indications of sex modification in the male direction. However, there are evidences in other amphibians that the male sex has actually become feminized. In Triton alpestris, Champy ('21) described a case of 'total sex inversion' from male to female. Caroli, among others, has noted that in toads certain parts of the testes sometimes develop in a female direction. Burns ('28) recently performed some transplantation experiments on Amblystoma, in which he removed a section of mesonephros, bearing the gonads, from a younger larva and inserted it into the body cavity of an older larva. His results showed that the male gonads of both graft and host were strongly modified: an ovarian cortex had appeared in the male graft by residence in a female host and, in at least one instance, in a male host through the presence of a large female graft. If we consider the sex condition in the frog as fundamentally similar to that in other amphibians, it seems logical, then, to assume that in frogs sex transformation toward femaleness might be a possibility.

In this connection, one should not neglect to consider the phenomenon of testis-ova,[9] which has, in some respects, an important bearing upon the process of sex transformation. Crew contends that in all cases where ova are found in the spermatic tissue of the testis portion of an ovotestis and of an otherwise normal testis, such ova are but the remains of previously existing ovarian tissue, which has been replaced by the spermatic tissue. This condition appears to be true in our specimen. In the left gonad of our frog, along the junctional region between the ovary portion and the testis portion, many ova from the former portion are being included in the latter portion and become gradually surrounded by the male germinal tissue. This phenomenon demonstrates the process of formation of the testis-ova. While contending for an ovarian origin of the testis-ova in our case, it should not be overlooked that in many other cases they are shown to be of testicular derivation. Meyns ('12) transplanted pieces of testis of one frog under the skin or into the body cavity of another. Such grafts, under favorable conditions, regenerated and the included germ cells developed into egg-like cells. Meyns concluded that these ovum-like cells were really ova and had arisen from male-determined germ cells. Guyénot and Ponse ('23) also observed in toads the occurrence of ova in testes when the latter were transplanted or were regenerating after incomplete castration. Dubois and Beaumont ('27) observed a similar phenomenon in Triton. Champy ('13) found in the adult testes of frogs, during the 'période interspermatogénétique,' oviform cells, which may develop further "et aboutit à la formation d'ovocytes incontestables qu'on ne peut distinguer de ceux d'un jeune ovaire." These facts are sufficient indication that testis-ova are not always of ovarian origin, but are in some cases produced by the modification of sex cells of the testes along the female line.

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[9] Testis-ova refer to ova or ovum-like bodies in an otherwise normal testicular tissue.

An account of hermaphroditism in frogs naturally recalls the phenomenon of Pflüger's hermaphroditism. In the so-called undifferentiated races the young frogs show distinct hermaphroditic tendencies, and sex is not definitely differentiated until late in development. It is conceivable that some of the earlier accounts on intersexual gonads of frogs are but descriptions of certain stages of Pflüger's hermaphrodites. But in Rana cantabrigensis, or at least in the Ann Arbor strain of this species, Pflüger's hermaphrodites do not occur and the sexes are distinctly differentiated at an early larval period. The occurrence in this strain of an adult hermaphrodite has, therefore, no relation to Pflüger's hermaphroditism and is, in whatever respect it may be considered, an abnormal phenomenon, while Pflüger's hermaphroditism is essentially a normal embryonic condition in certain races of frogs.

Lastly, it should be pointed out that in all abnormal hermaphrodites of frogs, including the one presented in this paper, which have been described as having undergone a sex transformation, or at least have been so interpreted, there is no knowledge whatsoever of the early history of the individuals. It is conceivable and probable that in these cases sex transformation may be initiated and induced by a defective sex differentiation in early embryonic development or by an abnormal hereditary mechanism of some kind. Crew makes a sweeping generalization when he says, "A gonad which previously had every character of a perfect ovary, has thus become replaced by a perfect testis," and again, "It is shown that the recorded cases of abnormality of the reproductive system illustrate the process by which an individual, at one time possessing solely the complete sex-equipment of the female, comes to exhibit the organization of the male." What evidence is there that these sexually abnormal frogs have ever possessed solely and normally complete sex organs of either one sex? Unless positive demonstration of this is furnished, we cannot speak of sex transformation of a normal frog in cases of abnormal hermaphroditism.

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THE EFFECTS OF THE SUBSTRATUM, DIVALENT AND MONOVALENT CATIONS ON LOCOMOTION IN AMOEBA PROTEUS[1]

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THREE FIGURES

AUTHOR'S ABSTRACT

The rate of locomotion in Amoeba proteus is dependent upon the nature of the substratum, the nature of the different divalent and monovalent cations present, and the ratio of the amount of the monovalent cations to the amount of calcium or strontium. Calcium is more efficient than strontium in antagonizing potassium. Magnesium and barium do not antagonize potassium. The four monovalent cations studied in antagonizing calcium are efficient in the following order Na > K > Li > Rb. Calcium and potassium are more effective against antagonistic cations when combined with chlorine than when combined with phosphate.

INTRODUCTION

The relation between the different factors in the environment and the life-processes, especially the process of locomotion in Amoeba, has long been a favorite subject for study and speculation by scientists interested in the fundamental life-processes of the cell. It is the object of the present paper to record the results of a study of the effects of four divalent cations (calcium, strontium, magnesium, and barium) and four monovalent cations (lithium, sodium, potassium, and rubidium) on locomotion in Amoeba proteus.

Edwards and Forgrave ('23) found that in solutions of single monovalent neutral salts the rate of locomotion varied with the concentration and with the nature of salts—the lower the concentration, the higher the rate—and that the rate of locomotion in each concentration decreased slowly as time passed, until it ceased altogether. Pantin ('26) maintains that a proper balance between calcium or strontium and a monovalent cation is necessary for locomotion in marine amoebae. Mast ('28) finds that Amoeba proteus is able to move freely in water of a purity as high as it is possible to prepare in a pyrex-glass still. This seems to indicate that salts are not necessary for locomotion in Amoeba proteus. In nature, however, we find that fresh-water amoebae always live in media containing varying amounts of different salts. What effects have these salts on locomotion? What are the relationships between the effects of these various salts? Do we find that certain cations of the salts act antagonistically to each other on locomotion in Amoeba proteus, as is the case in marine amoebae and in higher forms? This investigation is an attempt to answer some of these questions.

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MATERIAL AND METHOD

Amoeba proteus was used in these experiments. In fact, the same strain was used as in the observations described in my previous paper ('28). It was found desirable, however, to change the culture medium. The medium used in the previous work was not found to be satisfactory, for two reasons: In adding the hay to the medium a considerable amount of unknown inorganic salts was added, the dry weight of timothy hay being about 6 per cent inorganic salts; and the amoebae did not multiply rapidly, so that it was oftentimes difficult to obtain sufficient amoebae for experimentation.

For the purpose of eliminating these difficulties, the following medium was devised (Hopkins and Johnson, '29):

.00088 M. KOH
.0025 M. KH_2PO_4
.004 M. NaCl
.0002 M. $CaCl_2$
.2 gram dialyzed timothy hay per 100 cc. of solution

The hydrogen-ion concentration of this solution was pH 6.6. It remained fairly constant. The amoebae grew with greater rapidity and were more active in it than when raised in the medium used in the previous work. The constancy of reaction was also noticeably improved.

It was found, however, that great care must be exercised in keeping the volume constant by adding distilled water occasionally to make up for water necessarily lost by evaporation. In order to avoid error due to changing cultural conditions, each series of experiments was performed within a short period of time and on amoebae from the same culture. The same degree of precaution was taken in selecting and purifying chemicals and in cleansing glassware as was taken in my previous work and by Mast ('28).

Preliminary tests were performed and it was found that, if amoebae were placed into a mixture either of monocalcium acid phosphate and dicalcium acid phosphate having a hydrogen-ion concentration of pH 6.6 and a calcium concentration of 0.00678 N. or of monopotassium acid phosphate and dipotassium acid phosphate having a hydrogen-ion concentration of pH 6.6 and a potassium concentration of 0.00678 N., they ceased to move within one-half hour. But if potassium or any other monovalent cation was added to the calcium-acid-phosphate buffer or if calcium or strontium was added to the potassium-acid-phosphate buffer, they moved at a fairly uniform rate for several hours.

As a result of these preliminary experiments, the method was adopted of adding different concentrations of monovalent cations in the form of their chlorides to calcium-phosphate buffer, pH 6.6, having a calcium concentration of 0.00678 N.[3] and of adding different concentrations of divalent cations in the form of their chlorides to potassium-phosphate buffer, pH 6.6, having a potassium concentration of 0.00678 N.,[4] and then observing the rate of locomotion of amoebae in the different solutions. The concentration of the cation of the buffer was always 0.00678 N. Therefore, in these tests the cation in combination with the phosphate was constant, while the other varied.

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[3] The molecular concentration of the calcium-phosphate buffer was slightly less than that of the potassium-phosphate buffer; also, there may be a slight error in the amount of Ca in the calcium-phosphate buffers, due to the fact that the two substances $CaH_4(PO_4)_2$ and $CaHPO_4$ involved are difficult to get into solutions. However, the error, if it occurs, is not sufficient to cause an appreciable error in the results.

[4] Except in the study of the effects of the substratum, in which the buffer was reduced so that the potassium was 0.00339 N.

The rate of locomotion in a given solution was obtained, as in my previous work ('28), in the following manner: Five amoebae were removed from the culture, rinsed in three changes of the solution to be tested, allowed to remain in this solution fifteen minutes for adjustment; then the rate of locomotion was measured by successively making at minute intervals camera-lucida sketches of the posterior end of each of five amoebae as they moved along monopodally on the substratum. The average of five minutes' monopodal locomotion for each of five amoebae was taken as the rate in the solution. The rates of all five amoebae were measured within one hour.

THE SUBSTRATUM

In my previous work an ordinary glass substratum was used in the experiments. It was noticed, however, that unless the same dish was used throughout an experiment, very striking variations in the rate of locomotion occurred. Therefore, before undertaking a comprehensive study of the effects of the cations, different kinds of substrata were investigated, in order to obtain one which was of uniform composition and affected the locomotion of Amoeba uniformly.

Four different substrata were investigated: ordinary glass, ordinary paraffin, pyrex glass, and quartz. The dishes were all washed in potassium bichromate-sulphuric acid cleansing mixture and rinsed several times in redistilled water. Potassium-phosphate buffer, pH 6.6, was then added to each, and later five amoebae which had been washed in the buffer. In this solution containing no calcium, no locomotion was obtained in any of the dishes. Within one-half hour, all the specimens rounded up and never came out of this condition during the time of observation (one hour).

Similar observations were made with various amounts of $CaCl_2$ added to the potassium-phosphate buffer. The results obtained in these observations are presented in figure 1. The graphs in this figure show the following: On ordinary glass as the concentration of $CaCl_2$ increased the rate of locomotion increased very rapidly to a maximum; then it decreased, at first rather rapidly and then more slowly, until it became zero at 0.1 N. $CaCl_2$. On paraffin a very smooth curve was obtained. The rate increased rapidly at first, but not so rapidly as on ordinary glass. At 0.0002 N. $CaCl_2$ the rate was much lower than it was in the same solution on ordinary glass, but it continued to increase until a maximum was reached at 0.0006 N., then it gradually decreased until zero was reached at 0.1 N. On pyrex glass and quartz very similar curves were obtained, more regular in form than that on ordinary glass and slightly less regular than that on paraffin. Up to 0.0006 N. $CaCl_2$ the rate on pyrex and quartz was lower than it was on ordinary glass, but in higher concentrations it was very nearly the same. The rates for pyrex and quartz were uniformly much lower than for paraffin until a concentration of 0.01 N. $CaCl_2$ was reached, after which it was essentially the same for all.

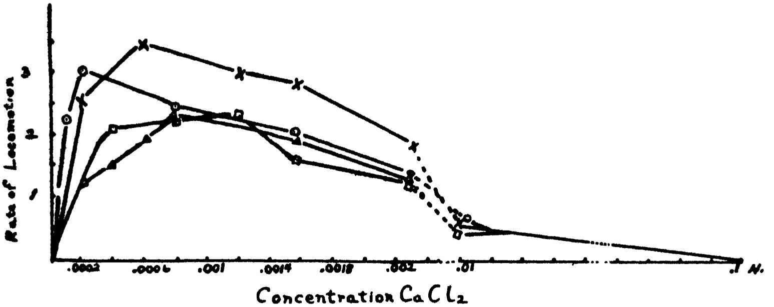

Fig. 1 Curves showing the relation between the nature of the substratum and the rate of locomotion of amoebae in $CaCl_2$ in potassium-phosphate buffer. ○, ordinary glass; ×, paraffin; △, pyrex glass; ☐, quartz. Rate in centimeters per minute × 140.

Ordinary glass, while variable in composition, is always a good substratum for attachment and locomotion, but paraffin is not. At one time uniform results may be obtained on paraffin—e.g., those given in figure 1—while at another time the amoebae may not move at all. This is probably due to variations in the treatment with the cleansing solution. Locomotion on pyrex glass and quartz is very constant as long as factors other than the substratum are not allowed to vary. It then at once becomes evident that of the substrata investigated, pyrex and quartz are best for the experiments outlined.

The variability of ordinary glass and paraffin is probably due to the solubility of substances present in them. It is known that ordinary glass is of variable composition and contains soluble sodium, potassium, and calcium compounds. The ordinary paraffin used probably contained some salt impurities and the action of the cleansing mixture probably changed its composition.

It should be stated here, in verification of these results with different substrata, that Mast ('28) finds that Amoeba proteus in pure water attaches and moves much more readily on ordinary glass than it does on paraffin, pyrex glass, or quartz. Thus we might infer that ions which facilitate locomotion diffuse from the soft glass, while they do not from paraffin, pyrex glass, and quartz. His results on paraffin are at variance with mine. However, he does not state whether he washed his paraffin with a cleansing solution or not. He probably used purified paraffin and then cleansed it with pure water. If this is true, the variation is to be expected, since water does not readily wet pure paraffin, while it does wet paraffin that has been treated with sulphuric acid-potassium bichromate cleansing fluid.

Pyrex glass was selected as a substratum for the following experiments, in preference to quartz, because it was much easier to obtain than quartz and because it was found that by evenly roughening the surface of the pyrex glass with emory paper a surface was obtained which exerted a constant effect on the amoebae and one to which the amoebae attached more readily than they did to smooth quartz or pyrex glass.

DIVALENT CATIONS

The study of the relation between the rate of locomotion of Amoeba proteus and the concentration of the divalent cations calcium, strontium, magnesium, and barium was now undertaken, using a roughened pyrex-glass substratum. The solutions of the chlorides of each of these cations were made up in potassium-phosphate buffer, pH 6.6, having a potassium concentration of 0.00678 N. Since amoebae do not move in this buffer solution alone, the rate of locomotion in these solutions is an index of the ability of the divalent cation of the chloride present to antagonize the monovalent cation, potassium, of the phosphate buffer. It was found that calcium and strontium only, of the four divalent cations, antagonize potassium efficiently. The results obtained with these are presented in figure 2. In this figure it is to be observed that the addition of a very small amount of strontium or calcium causes a marked increase in the rate of locomotion and that as the amount added increases the rate increases very rapidly to a maximum, after which it decreases very slowly at first, then more rapidly, until locomotion ceases at or before 0.1 N. However, in concentrations between 0.0001 N. and 0.002 N. there is but little difference in rate in either $CaCl_2$ or $SrCl_2$. The highest rate is obtained using

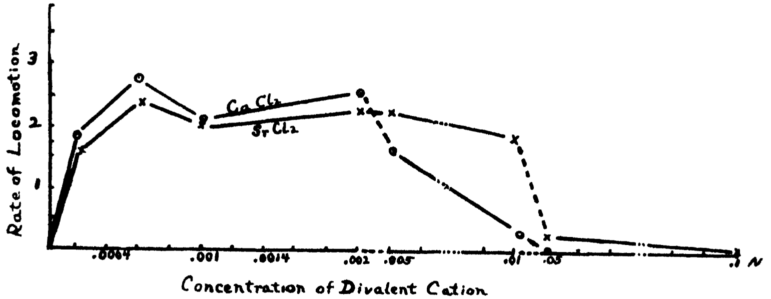

Fig. 2 Curves showing the relation between the rate of locomotion and the concentration of strontium and calcium in potassium-phosphate buffer, pH 6.6 (K, 0.00678 N.). Rate in centimeters per minute × 140.

$CaCl_2$ at a concentration a little less than 0.001 N. In the lower concentrations the rate in the two series of solutions is nearly the same. In the higher concentrations the rate in strontium solutions is uniformly higher than in calcium.

No locomotion was obtained in either the magnesium or the barium solutions. However, the forms assumed in these solutions differ from that assumed in the buffer solutions. The amoebae in the magnesium and barium solutions throw out long slender pseudopods which are fairly permanent and in which little protoplasmic streaming is to be observed, while in the buffer alone there are few, if any, pseudopods formed.

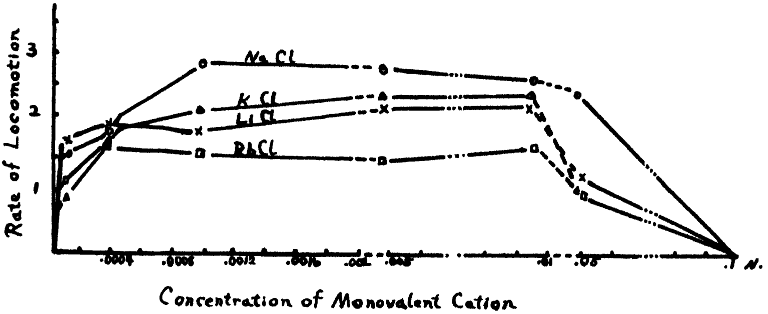

Fig. 3 Curves showing the relation between the rate of locomotion and the concentration of different monovalent cations in calcium-phosphate buffer, pH 6.6 (Ca, 0.00678 N.). Rate in centimeters per minute × 140.

MONOVALENT CATIONS

The relation of the monovalent cations to the rate of locomotion was studied in the same way as that of the divalent ones, except that the solutions were made up in calcium-phosphate buffer, pH 6.6, having a calcium concentration of 0.00678 N., instead of potassium-acid-phosphate buffer. The results are presented in figure 3. The general form of the curves in this figure is the same as that of those for calcium and strontium. It is to be noticed that in the lower concentrations all four of the curves run very close to each other, but that in the range of maximum rates they separate markedly. The maximum rates in the sodium solutions are the highest, then comes potassium, lithium, and finally rubidium. The lithium curve runs along very close to that of potassium. Except for lithium, the maximum rates obtained in solutions of these cations are in the reverse order of their atomic weights and their chemical activity. The order of activity and atomic weights is Rb>K>Na>Li, while the order of maximum rates attained in the solutions of the cations is Na>K>Li>Rb.

DISCUSSION

Optimum monovalent-divalent cation ratios

From the above results it is evident that the rate of locomotion in Amoeba proteus is correlated with the ratio between the divalent and the monovalent cations in the surrounding medium, but that the ratio for optimum locomotion is indefinite. The optimum monovalent-cation / divalent-cation ratio calculated from the data given in the curves of figures 2 and 3 (using the normal concentration in the calculations) varies as follows:

1. Potassium combined with phosphate, calcium with chlorine: .00678/.0006 to .00678/.002 = 11.3 to 3.4.
2. Potassium combined with phosphate, strontium with chlorine: .00678/.0006 to .00678/.01 = 11.3 to .67.
3. Potassium combined with chlorine, calcium with phosphate: .001/.00678 to .01/.00678 = .15 to 1.8.
4. Sodium combined with chlorine, calcium with phosphate: .001/.00678 to .05/.00678 = .15 to 7.4.
5. Lithium combined with chlorine, calcium with phosphate: .0004/.00678 to .01/.00678 = .06 to 1.5.
6. Rubidium combined with chlorine, calcium with phosphate: .0004/.00678 to .01/.00678 = .06 to 1.5.

It is interesting to note that the values of this ratio for most of these mixtures tend to vary around 1.0 as a mean.

The calculations just made show that when potassium is combined with phophate and calcium with chlorine, calcium is far more effective in antagonizing potassium than when calcium is combined with the phosphate and potassium with chlorine. This indicates that the phosphate in some way tends to hold the cation with which it is combined from action on or in the amoebae. It is possible that the large phosphate anion hinders penetration of the cation into the amoebae.

The nature of antagonism

How do divalent cations antagonize monovalent cations in their effect on locomotion? Chambers and Reznikoff ('26) have shown that if KCl or NaCl is injected into amoebae, the protoplasm becomes fluid, the amoebae becoming rounded up, and that the same results are obtained if amoebae are immersed in pure solutions of NaCl or KCl. On the other hand, they find that $CaCl_2$ or $MgCl_2$ injected into amoebae causes gelation, but that when amoebae are immersed in pure solutions of either of these salts, gelation does not readily occur. From these results they conclude that NaCl and KCl readily penetrate the amoebae, while $CaCl_2$ and $MgCl_2$ do not. They also state that calcium serves to stabilize the membrane, but they do not say whether calcium and magnesium can in time penetrate the membrane of the amoebae.

Mast ('26) describes amoebae as having a gelated layer of protoplasm just under the plasmalemma which he calls the plasmagel, while the central portion of the protoplasm, called the plasmasol, is solated. The fact that the outer layer of the protoplasm is gelated indicates that these gelating cations, calcium etc., do to a certain extent penetrate the membrane. The rôle of the divalent and monovalent cations in locomotion then appears evident. The monovalent cations, penetrating readily, reach the interior in far greater quantities than the divalent cations, hence the fluidity of the interior is increased. In the exterior layer both monovalent and divalent cations are present; however, the divalent cations are present in this layer in sufficient quantities to increase its viscosity. The higher the concentration of monovalent cations, the more solated the protoplasm becomes, and the higher the concentration of the divalent cations, the more gelated it becomes. If this is true, a mixture of mono- and divalent-cation salts in the surrounding medium results in a decrease in viscosity from the surface inward. If a definite amount of rigidity in the outer layer of protoplasm is necessary for optimum locomotion, it is evident that if this layer becomes too rigid, due, for instance, to the presence of too much calcium in the external medium, locomotion is hindered, and that if it becomes too fluid, due to the presence of a high concentration of monovalent cations, locomotion is also hindered. If all this obtains, then, other factors being constant, the highest rate of locomotion is obtained when the monovalent cation-divalent cation ratio produces an optimum ratio of gelation to solation.

Variations in the effects of individual monovalent and divalent cations

It should be noticed that the maximum rates attained in the solutions of monovalent cations balanced against calcium buffers and in the solutions of divalent cations balanced against potassium buffers tend to vary with the atomic weights of the cations. The greater the atomic weight, the smaller are the maximum rates attained. Higher rates are obtained in calcium chloride-potassium phosphate buffer mixtures than in strontium chloride-potassium phosphate buffer mixtures. With the monovalent cations balanced against calcium-phosphate buffers, the maximum rates obtained vary in the following order: Na>K>Li>Rb. All but lithium come into their logical place in the series. Reznikoff ('28) has shown that when mixtures of $CaCl_2$ and KCl or LiCl are injected into the amoebae, lithium is thirty-two times as effective in antagonizing calcium as potassium or sodium. Therefore, it seems reasonable to conclude from Reznikoff's and my experiments that LiCl penetrates the amoebae and solates the protoplasm much more effectively than KCl or NaCl. This would explain the failure of lithium to come into its proper place in my series. It should be noted (fig. 3) that in the lowest concentration used in any experiments, lithium gives a higher rate than either potassium or sodium.

SUMMARY

1. Amoeba proteus is sensitive to the chemical composition of the substratum. The rate of amoeboid locomotion is dependent upon the nature of the substratum.

2. In the presence of a relatively high concentration of calcium or strontium, locomotion in Amoeba proteus is impossible unless monovalent cations are present.

3. In the presence of a relatively high concentration of a monovalent cation, locomotion in Amoeba proteus is impossible unless calcium or strontium is present.

4. Calcium antagonizes potassium more efficiently than does strontium, but strontium is less toxic in higher concentrations.

5. The efficiency of the monovalent cations in antagonizing calcium is in the following order: Na>K>Li>Rb.

6. Magnesium and barium are not effective in antagonizing monovalent cations in reference to locomotion in Amoeba proteus. They do, however, prevent liquefaction.

7. The antagonizing efficiency of a given cation is not so great when it is combined with a phosphate anion as it is when it is combined with a chlorine anion.

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PERIODIC REVERSAL OF HEART ACTION IN THE SILKWORM MOTH AND PUPA[1]

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FOUR TEXT FIGURES AND ONE HELIOTYPE PLATE

AUTHOR'S ABSTRACT

Direction of peristalsis of the dorsal vessel in prepupa, chrysalis, and adult of Lepidoptera is not constantly forward; phases of forward peristalsis alternate with phases either of backward peristalsis throughout the whole dorsal vessel or of diverging waves originating in the third to fourth abdominal segments, proceeding backward and forward. Periodic reversal in the silkworm begins twenty-four hours after spinning, forty-eight before pupation. Pupation occurs during a vigorous backward phase. Soon after pupation, and sometimes during pupation, backward phases consist of diverging waves. Later, and in the adult, complete reversal intermittently occurs. Backward phases in the pupa are long (e.g., fifteen minutes), the average rate slow and variable, quickening at eight to ten days to that of the adult. Any long backward phase shows a gradual slowing down, except approaching pupation when the pulse quickens to equal that of forward beating. Average rate of forward beating is constant at constant temperature, but absolute rate in a long phase gradually slackens. Pauses (sometimes one and one-half hours long in older pupae) often follow forward phases Alternating phases in the adult, compared with the pupa, are short, about one minute and usually less than one hundred beats. In old moths the phases become still shorter, often being reduced to single beats Opposing waves occasionally conflict. Periodic reversal occurs for hours after excision of the dorsal wall with the pericardium and heart. As in ascidians, back pressure cannot explain reversal.

CONTENTS

Introduction 386
The unity of the insect heart 388
Methods 393
Reversal in the mature larva, after spinning 394
Pupation 395
  Table 1. Reversal just before pupation 396
  Table 2. Reversal during pupation 397
  Résumé 399
The pupa 399
  Diverging waves, or double-action (table 3) 399
  Thirty to sixty minutes after pupation 400
  One and one-half to four hours after pupation 402
  Twelve to twenty-four hours after pupation 403
  Rate of pulsation during the pupal stage 404
  Changes in rate during single phases 406
  Pauses 407

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Periodic reversal in the adult moth 407
  Table 4. Young non-mating moths 410
  The effect of mating on periodic reversal (table 5) 411
  General conclusions regarding the effects of mating 411
  Long phases in the male 413
  An exceptional female 414
  Variations accompanying old age 414
  Temperature 417
Explanation of periodic reversal 419
  Similarities between periodic reversal in Lepidoptera and Ascidia 419
  Theories as to the cause of periodic reversal 421
Summary and conclusions 425
Literature cited 428

INTRODUCTION

Periodic change in the direction of peristaltic heart action is no longer to be regarded as a unique characteristic of ascidians. I have abundant evidence showing that Lepidoptera, upon reaching the prepupal stage, acquire the same remarkable type of rhythmic action as that found in ascidians, and my recent observations prove that it continues throughout adult life.

The story of the discovery of this phenomenon, beginning with Malpighi's (1669) observations on the silkworm moth, told in another paper (Gerould, '29 b), is an extraordinary instance of the slow progress of science in regard to some most-ubiquitous processes. For nearly 260 years his notable observations of periodic reversal of heart beat in the adult moth remained unconfirmed, though frequently questioned, while his incorrect conception of the dorsal vessel of Lepidoptera as a series of chambers or little hearts ('corcula') persisted.

The present paper is a record of the observations upon Bombyx mori made during the summers of 1927 and 1928, supplemented with less detailed studies on Telea polyphemus, Actias luna, Samia cynthia, Sphinx chersis, Ctenucha virginica, and the wingless female of Notolophus leucostigma. In these and other moths periodic reversal may be seen without vivisection. In butterflies, e.g., Argynnis aphrodite, I have had to resort to Malpighi's method of cutting open the body wall—a much less satisfactory method than the study of the denuded but otherwise normal insect.

Besides these studies of the adult, I have observed periodic reversal in other species of butterflies and moths in the pupa. The same has been done independently by Dr. E. Fischer ('18), the eminent entomologist of Zürich, whose discovery of it in the pupa antedated mine (Gerould, '24). In fresh chrysalids of butterflies of the genus Colias both of us have found it particularly striking, though fresh chrysalids of the American species, C. eurytheme, C. philodice, are more translucent and favorable for observation than those of the European C. hyale.

The universal occurrence of periodic reversal in Lepidoptera leads me to expect that it will be found in other orders of holometabolous insects. Kirby and Spence, just a hundred years ago (1828, vol. 4, p. 97), called attention to something similar in an adult syrphus fly (Syrphus pyrastri) (Gerould, '29), but I have not been able to repeat or confirm their observations.

Bataillon ('93) was the first to show the connection between periodic reversal and metamorphosis and to point out exactly when in the life history of the silkworm the continuous forward peristalsis of the larval dorsal vessel begins to be periodically interrupted by backward beating.

Although periodic reversal is not, as Bataillon at first concluded, a temporary phenomenon characteristic of the prepupa and pupa, it is a significant feature of metamorphosis. Backward beating of the dorsal vessel during pupation, combined with the probable valve-like action of the constricted base of the abdomen preventing the forward flow of blood in the ventral sinus, results in the swelling of the abdomen, which assists in throwing off the last larval skin.

A more important function of direct backward flow, however, is the better irrigation of the ovaries during their rapid growth in the pupa.

Of greatest interest are the changes in the average rates of pulsation, especially in backward beating, which are described in detail in this paper. The speeding up of backward beating just before pupation and its extraordinary variability during the first two or three days of the pupal stage are probably the index of important chemical changes in head and thorax where the largest amount of regulatory changes in the whole body are going on, in connection with metamorphosis of the appendages.

Of great interest, too, is the gradual slowing down of the rate during any long phase,[2] whether backward or forward, like a chemical reaction fading out. Probably the ganglia which presumably start the waves of peristalsis are being acted upon chemically by substances of limited quantity or chemical mass action is lessening the stimulating effect.

To keep this paper within proper limits I am omitting tables showing the observations in detail, averages of these data being recorded in the graphs (figs. 3 to 6). The original records show a remarkable irregularity in the duration and the number of beats in almost any series of forward or of backward phases. At first sight, there would seem to be no law about the pulsation, and one would be inclined to agree with Malpighi that nothing could be more variable, but study of the averages at successive periods of the life history reveals, as the graphs show, considerable order. The heart action during each period has its own peculiar features, depending upon the internal chemical and physical conditions to which the insect in its various parts is subject at that stage.

THE UNITY OF THE INSECT HEART

If the standard description of the dorsal vessel in insects current in even the most recent text-books of entomology is accurate, one would conclude from a study of the observations recorded in the present paper that either the silkworm and other Lepidoptera have a somewhat different type of heart from that of other insects or that certain errors have crept into the prevailing description of the dorsal vessel, which states that the heart contains a series of chambers, separated by valves past which the blood can flow only in one direction, that is, forward.

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[2] Except the backward phase including pupation, in which there is an equally gradual acceleration.

One of the more accurate text-book descriptions is that of Folsom ('22):

> The dorsal vessel is a delicate tube extending along the median-dorsal line immediately beneath the integument. A simple tube in some larvæ, it consists in most adults chiefly of a series of chambers, each of which has on each side a valvular opening, or *ostium*, which permits the ingress of blood but opposes its egress; within the chambers occur other *valvular folds that allow the blood to move forward only.* With few exceptions (Ephemeridæ) the dorsal vessel is blind behind and the blood can enter it only through the lateral ostia. *Aorta*—The posterior, or pulsating portion (*heart*) of the dorsal vessel is confined for the most part to the abdomen; the anterior portion, or *aorta*, extends as a simple attenuated tube through the thorax and into the head, where it passes under the brain and usually divides into two branches, each of which may again branch. In the head the blood leaves the aorta abruptly and enters the general body cavity.

As a brief description this is accurate in most respects. It correctly states that in some larvae it is a simple tube, but claims, 1) that "in most adults" the abdominal portion consists of a series of chambers, between which are, 2) "other valvular folds that allow the blood to move forward only."

These two statements which may be open to criticism are illustrated by Straus-Dürckheim's (1828), Kolbe's ('93) figures.

Straus-Dürckheim's description of the heart was strongly criticized by Verloren ('44), whose painstaking investigations and admirable figures show that the insect heart, as he saw it, is not formed of compartments separated from one another by valves.

> Il faut regarder la partie cardiaque comme ne formant qu'une seule cavité, ainsi que l'a fait très-justement Réaumur. J'ai déjà refuté aussi la forme variqueuse que Malpighi avait attribuée au vaisseau dorsal.

On the other hand, Kolbe's figure of the heart of a dragon-fly larva, Epitheca (Folsom, '22), illustrates the conception of the heart as consisting of distinct chambers, each separated by pairs of 'interventricular' valves in addition to those of the ostia. Zawarzin ('11) also adopted this view in his studies of the histology of the heart in the larva of Aeschna and figures such valves opposite the divisions between body segments.

Popovici-Baznoşanu ('05), after recording observations on the larvae of Chironomus, Clio, and other Diptera and giving a very thorough historical review of the conflicting views on this subject, came to the following and other conclusions: 1) The heart of insects is not chambered, but is a single tube, in which the appearance of separate chambers is an illusion produced by the striking together of the ostial folds. 2) The 'interventricular' valves of the Chironomidae represent the anterior ostial folds of the larvae of Ephemeridae. Questioning the accuracy of Kolbe's description and figure of regularly alternating ostia and 'interventricular valves' in the dragon-fly, Epitheca bimaculata, he says:

> Wo sog. Interventricularklappen bei Insekten vorkommen, wie bei den Larven von Chironomiden und Ephemeriden, sind sie Ostialfalten, die nach Verschluss der zuhörigen Oeffnungen übrig bleiben und nunmehr noch als Ventile wirken. In der Regel fehlen aber sog. Interventricularklappen ganz. . . . Aber zur Bildung von besonderen, die Herzkammern gegeneinander abgrenzenden Klappen kommt es nicht.

Thus he admits that paired valves projecting forward into the lumen of the heart really exist, derived originally from ostial valves, but denies that they mark the limits of special metameric 'chambers,' as Graber and as Kolbe supposed and as Zawarzin ('11) later claimed.

Snodgrass ('25), in his excellent treatise on the honeybee, follows the common custom of mentioning 'heart chambers,' though his figures show only slight undulating constrictions between them, and he remarks: "There are no special valves between heart chambers as formerly stated."

The heart of the silkworm has been studied at various stages in its development by Verson ('08), who finds that it is a simple muscular tube without preformed chambers and with no valves or partitions dividing its cavity. The dilatations and constrictions observed after death in fixed specimens are irregular, not segmentally arranged, varying in position and shape from one period of development to another. Six pairs of ostia were found by Verson, each a little in front of an intersegmental region.[3] Each ostium resembles a bottomless pocket extending forward from the external (pericardial) opening, which is so narrow a slit, according to Verson, that leucocytes cannot pass through, as they are seldom or never found within the dorsal vessel.

Ostia are not clearly visible in dissections or cleared specimens, but Verson investigated their structure at various periods of embryonic and larval development by means of transverse and frontal sections of the dorsal vessel. It is readily seen by Verson's figures (figs. 1, 2) that the lateral walls of the dorsal vessel are so far apart in mature larvae and in pupae that ostial valves on opposite sides of the tube cannot reach across the lumen to occlude the passage and prevent backward flow of the haemolymph.

In form and in position, as shown by figures 1 and 2, they are roughly comparable to the spiracles of a shark in reference to the pharynx. In diastole they open, and the fluid passes obliquely forward and inward into the lumen; in systole they close by flattening against the lateral wall of the tube, and so prevent the haemolymph from regurgitating into the pericardium, *but they can have no effect whatever upon the backward or forward flow of blood in the dorsal vessel.*

Thus, in Lepidoptera, an ostial valve consists of a single membranous flap extending inward and forward from the ostium; in larval Diptera, however, a flat funnel consisting of two flaps (anterior and posterior) projects inward and forward from the pericardial opening into the lumen of the tube far enough so that in some cases the anterior (or posterior) flap may touch the corresponding member on the other side and presumably prevent backward flow within the tube. Such contact and closure of the lumen, as Verloren ('44) long ago pointed out, could take place only during diastole while the ostia are wide open. During systole, when the ostial valves close, their projection into the lumen, he thought, would favor forward and impede backward flow. His idea that the cardiac cavity acts as a single compartment, all the ostia opening at practically the same moment and closing simultaneously as the wave of peristalsis passes forward, seems more plausible than the opinion of Malpighi, Straus-Dürckheim, Graber, and Kolbe that each supposed 'chamber' acts singly in succession.

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[3] Verloren ('44) found eight pairs of ostia in the larva of Sphinx ligustri.

The conception of the heart as a single physiological unit is strengthened by the discovery of Zawarzin ('11), in the dragon-fly Aeschna, and by Alexandrowicz ('26), in the cockroach Periplaneta, of lateral nerve trunks running the length of the heart and uniting at its caudal end.

From these somewhat discordant data we may venture to draw the conclusions: 1) That the insect heart is typically a simple tube, not a chain of separate compartments, as Malpighi and many of his followers have claimed; 2) that the heart, like the intestine, acts in peristalsis (and antiperistalsis) as a unit, not as a chain of 'chambers' acting independently in succession; 3) that in Bombyx and other Lepidoptera no internal valves occur in the dorsal vessel which might impede the backward flow of the blood, that in the pupa and imago the lateral ostia are inconspicuous and relatively minute; 4) that in the honeybee no internal or 'interventricular' valves exist; 5) that in the larvae of Odonata, Ephemerida, and certain aquatic Diptera ostia are found only near the posterior end of the tube, being represented anteriorly by segmentally arranged valvular folds possibly tending to impede backward flow; 6) that too little is yet known about the heart and circulation in adult insects of the orders just mentioned to be able to assert that the flow of the blood in the dorsal vessel is constantly forward.

METHODS

Mature larvae removed from the cocoon on the second day after spinning begin to show periodic reversal, and on the third day pupation occurs.

Peristalsis of the dorsal vessel may be observed by any hand lens, but for long-continued and precise observation a binocular dissecting microscope is indispensable. An assistant[4] with a stop-watch notes the number of beats in each successive phase as counted aloud by the observer and the length of each phase in minutes and seconds. He measures and records pauses between beats when they exceed in length that demanded by the rhythm, conflicting pulsations when they occur, and other striking variations. To show the changing rate during a single phase, the number of beats in each ten seconds was often recorded. For greater accuracy, the observer should operate a stop-watch himself, handing it to a second assistant to read and keep in readiness.

Observations on the moth, especially of the female, can be made even more satisfactorily than on the pupa. The scales of the back are rubbed off easily with a small swab of cotton or paper, followed by scraping and picking with forceps. The chitinous tergites are transparent, and the blood, in individuals of the race with which I have been working, slightly colored, so that the outlines of the dorsal vessel can clearly be seen with a binocular dissecting microscope; the direction of circulation in the moth is visible without a lens. The whole length of the dorsal vessel, from the median aorta of the prothorax and the pulsating mesothoracic vessel backward to the posterior end of the abdomen, can be readily observed in the female, whereas in the male opacity of the body wall in the posterior half of the abdomen restricts observation to the mesothorax and the first four or five abdominal segments. Injections of India ink (rubbed from a stick into tap-water or normal salt solution), of carmine granules, or of methylene blue into the ventral sinus are useful in studying morphological details, and the flow of blood through the sinuses can in this way be studied. The heart remains active a long time under this treatment, unless too much water is injected, and periodic reversal still can be seen.

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[4] Messrs. George F. Vaughan and A. Porter Haskell, Jr., served as assistants.

The numerical data given in this paper, however, were taken directly from the uninjured insect as observed with a Leitz stereomagnifier.

Excellent preparations showing the live heart in position can be made by making two parallel dorsolateral incisions along the length of the back just lateral to the pericardium and a cut across the last abdominal tergite posterior to the heart. The dorsal region, thus isolated, can be lifted from the underlying tissues, inverted, pinned out upon a strip of cork, and examined upon its inner or ventral surface. Such a preparation will retain its vitality and show periodic reversal of peristalsis for several hours if kept in a moist chamber.

REVERSAL IN THE MATURE LARVA, AFTER SPINNING

The pulsations of the heart or dorsal vessel of the silkworm up to the time of spinning the cocoon run forward from the posterior end, as in insects generally. Pupation, or the shedding of the larval skin, takes place on the third day after spinning. If one cuts open the cocoon on the first day after spinning, the heart of the larva is found still to be beating forward. On the second day after spinning, however, the mature larva (prepupa) has undergone striking physiological changes. Forward beating is no longer continuous, but is interrupted at more or less regular intervals by phases in which pulsations run backward through the whole dorsal vessel.

This prepupal alternation in the direction of pulsation continues during the forty-eight-hour period (more or less) immediately preceding the last larval molt or 'pupation.' The beats of the forward phases are then, as later, almost invariably more rapid than of the backward; the average length of one beat in five larvae was 1.8 seconds forward against 3.5 seconds backward. Each backward phase is on the average longer than the previous or succeeding forward phase, the average backward phase before pupation, according to my data of 1927, lasting 7 minutes 25 seconds—nearly twice the average forward phase, which is 3 minutes 55 seconds (fig. 3).

The average number of beats in one phase is at this time slightly over 100. My data show a somewhat larger number of beats per phase in forward than in backward pulsation (123 vs. 104; fig. 5), the range of variation even in the same individual being very large.

PUPATION

Circulation through the 'heart,' or abdominal dorsal vessel, during the act of pupation is invariably caudad. In all seven individuals in which shedding of the last larval skin was observed the beating was exclusively inverse, either wholly backward throughout the whole dorsal vessel, including the aorta, or, in about an equal number of individuals, it was what may be called 'double action,' backward from the junction of the third and fourth abdominal segments and simultaneously forward through the first three abdominal segments and the aorta. In other words, diverging waves proceed caudad and cephalad from about the middle of the dorsal vessel as in 'central beating' in ascidians (Hecht, '18). The backward abdominal stream, therefore, in these individuals comes not from the aorta, but has its source at this time exclusively from the ventral sinus.

The dorsal vessel during pupation becomes distended, literally gorged with haemolymph; its intense activity is also shown by the fact that the rate backward (1 beat in 1.5 to 2 seconds) is at its maximum and has reached that of forward beating. Much blood from the ventral sinus flows directly upward in the median plane at the junction of the third and fourth abdominal segments, through a temporary opening among the tissues, as well as through the usual lateral channels. As I expressed it in the case of the butterfly Colias (Gerould, '24 a, p. 5), the blood "wells up like a fountain into the pericardial sinus.'' One who is skeptical as to the reality of backward flow or of its importance in ridding the body of the larval skin needs only to watch the act of pupation with a stereomagnifier to be convinced.

The most favorable of the seven or more opportunities which I have had to observe the process was in the silkworm prepupa 't ♀,' on August 9, 1928, in which the phase including pupation lasted over an hour and a half. During this period peristalsis and circulation in the abdominal portion of the dorsal vessel (except the first three segments) was backward.

Immediately preceding this extraordinarily long backward phase there was a very gradual slowing down of the average rates in the series of forward phases and a gradual speeding up in the backward phases. The result was that rate of alternating forward and backward phases became equal (table 1). A similar change has been observed in several individuals. It is typical. The gradual acceleration observed in the averages of preceding backward phases was continued during the course of the long backward phase itself. At the beginning (11.24 A.M.) the rate was 10 beats in 24½ seconds; an hour later (12.25 P.M.), it had very gradually become accelerated to 10 beats in 16 seconds. Three minutes later,

TABLE 1

Periodic reversal of heart beat in a larva just before pupation

FORWARD				BACKWARD			
	Time				Time		
Beats	Minutes and seconds	Seconds	Average rate, 1 beat, seconds	Beats	Minutes and seconds	Seconds	Rate, 1 beat, seconds
				→ 55	2.36	156	2.8
→ 34	1.0	60	1.7	57	2.24	144	2.5
→ 35	1.0	60	1.7	65	3.0	180	2.7
→ 35	1.12	72	2.0	64	2.36	156	2.4
→ 34	1.12	72	2.2	60	2.24	144	2.4
→ 31	1.12	72	2.3	1661	57.0[1]	3420	2.0
			Rate retarded				Rate accelerated

[1] Count broken off at 57 minutes. This phase continued through pupation. (Observed 1 hour 33 minutes.)

when the skin began to be stripped off at the posterior extremity of the body, the rate had reached 10 beats in 14 seconds. A spurt of still greater activity occurred just preceding the bursting of the skin along the dorsum of the thorax, when the rate momentarily reached its maximum of 10 beats in 10 seconds (12.41 P.M.). Soon after the skin had actually burst, six minutes later (12.47 P.M.), the rate fell slightly to 10 beats in 17 seconds, but soon rose again to 10 beats in 13 or 14 seconds during the last two minutes of the act of pupation.

TABLE 2

Pulse rates (seconds per 10 beats) during an extraordinarily long backward phase before, during, and after pupation. Temperature, 74°F. = 23°C. The first part (11.24 A.M. to 12.21 P.M.) covers the last partial phase given in table 1. The latter part continues this phase, including samples of 10 beats taken up to, during, and after pupation. Note the gradual acceleration from 10 beats in 24½ seconds (at first) to 10 beats in 10 seconds after pupation begins. No reversal to beat forward occurred for over an hour and a half

11 24 A M										
24½	23	23½	22	22	22½	23	22½	22½	23	22
23	24	22½	22½	22	22½	22	21¼	21¼	21½	21
								11 47½ A M		
21	21⅗	21⅗	21¼	22	21	21	21½	21	21⅗	21
								11 55		11 57 A M
21	20⅗	21	21	21	21⅗	20⅘	21	20	21⅗	22½
21	20	20½	20½	20	20	21	20	22	21½	20
21⅗	20	19	20	20	19⅗	20	19	18	20	19
19	19	18	19	19	19	19½	17½	19	17½	18
					12 20 P M				12 25 P M	
17⅜	18	18	17½	17½	17 (1661 beats in 57 min.)				16	15⅘
15	15	14½	15	14	13⅘	14	14	14	14	13½
		12 31 P M							12 34½ P M	
14½	14	14	14	13	14	13⅗	13½	14½	14	14
						12 38				
14	14	14	13⅗	14	14	13⅘	14	13⅗		
	Shedding begins (tail)									
14	14	14	10	11	11	13¼				
	Skin bursting, thorax exposed									
			12 47				12 51, skin almost off			
14½	14	14½	16½	17	15½	15	13½	13	13	13½
		12 53 P M								
13½	13	14	End of pupation							

12.54 ... 12.57 P M

Pupa 13⅜ 14½ 15 13½ End of observation; backward beating still continues (pronounced 'double action').

These variations indicate, it seems to the writer, that, whereas the velocity of beating is probably regulated chiefly by chemical conditions acting upon the neuromuscular mechanism and inducing a high blood pressure in the vessel, as shown by its distention and that of the whole thorax, changes in the mechanical resistance to this pressure offered by the stretched and bursting skin probably account for some of the minor variations in rate.

Observations on other individuals during pupation showed always, as already stated, only long-continued inverse beating. In 1928, no. p♀ pupated from 8.22 to 8.33 in the evening. During this time and for 20 minutes afterward, peristalsis was completely backward, with an average rate of 10 beats in 18 seconds. A short forward phase of less than 2 minutes followed. A half-hour after pupation (9 P.M.), the inverse phases were for the first time 'double action.' (Temperature, 70°F.)

Individual 1928 no. q pupated at noon (12.13 to 12.16 P.M.), temperature 72°F., in the midst of a long phase of double action which lasted at least 15 minutes after pupation was completed (observation interrupted at 12.32). The rate varied from 10 beats in 18 seconds before and during pupation to 10 in 15½ seconds twelve minutes after its completion.

Completely backward pulsation in 1928 no. 4 continued for 30 minutes after pupation before forward pulsation began, the first phase of which lasted for only 27 seconds (30 beats). It was followed by a relatively short inverse phase of only 104 seconds (56 beats).

In another individual (no. 5) inverse pulsation (double action) lasted throughout pupation and for 1 hour 2 minutes afterward, broken only by an attempted reversal to beat forward at 44 minutes 20 seconds from the end of pupation. In this 'attempt' peristalsis started at the posterior end of the dorsal vessel and met the advancing backward waves. This phase, the conflicting beats of which were not counted, lasted for 1 minute 35 seconds. The first successful reversal to beat forward lasted only 1 minute 4 seconds (45 beats) at the rate of one beat in 1.4 seconds.

Résumé

1. Pupation occurs during a backward phase, the average rate of which is as rapid as that of forward beating.

2. This phase in some individuals is wholly backward, in others 'double action' (= central beat of ascidians).

3. It lasts in certain individuals over an hour without interruption and was observed in one case for over an hour and a half.

4. When this backward phase is long continued, the rate during the phase becomes gradually accelerated, reaching a maximum during ecdysis or shortly afterward.

5. In the alternating phases just previous to that in which pupation occurs, the average rates forward were gradually retarded, those backward were accelerated till both were equal.

6. Inverse pulsation assists the pupa in sloughing off the larval skin. The rapid flow of blood caudad dilates the walls of the abdomen and stretches the integument, while by muscular action the skin is being sloughed off backward.

THE PUPA

Diverging waves, or double action

The tendency to send out diverging waves from the third-fourth abdominal segments, though a transitory modification of backward beating, is very strong immediately after pupation. All seven individuals on which I have notes showed this type of circulation. The upward flow from the ventral sinus near the base of the abdomen is apparently so vigorous as to prevent reversal in the aorta. Just how long this lasts is not shown by my data, as I did not attend specifically to this point, but in one individual (1928, no. q) it still continued at 3½ hours after pupation. In another pupa (1928, no. p♀) at 14½ hours, however, it had given way to completely backward peristalsis, which is characteristic of inverse beating in the adult. Table 3 summarizes the observations.

TABLE 3

	BEFORE PUPATION	DURING PUPATION	AFTER PUPATION	OLDER PUPA
s♂	C	Not observed	D (seen 15 minutes after pupation)	
p♀	C	C	D (began 30 minutes after pupation)	C (at 14½ hours)
q	C	D	D (3½ hours)	
t♂		D	D	
(4)♂		C	D	
(5)		D	D	

C, completely backward; D, double action.

Thirty to sixty minutes after pupation

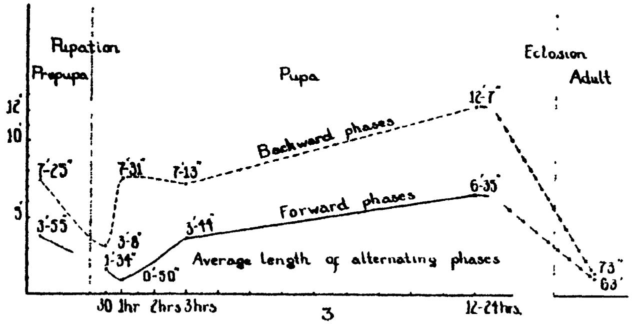
Thirty minutes after pupation, the average length of inverse phases has about the same relation to that of the forward phases as before pupation, viz., the inverse are twice as long (fig. 3), but the rapidity of pulsation, as measured by the average length of a single beat (fig. 4), shows less difference (1.7 seconds backward, 1.18 seconds forward). Both are at maximum rapidity. Naturally, the average number of beats in the long inverse phases is much greater than in the shorter forward phases (110 vs. 79) (fig. 5). These conditions soon change, however.

At one hour after pupation in the observations of 1927, the average length of the inverse phases increased to prepupal dimensions, 7 minutes 31 seconds, while that of the forward phases fell to a minimum of 50 seconds. The average pulse rate (or interval between successive beats) meanwhile

Fig. 3 Average length of alternating phases, in minutes and seconds (ordinates), at successive hours after pupation (abscissas). The solid line represents forward beating (interrupted during pupation); broken line, backward beating. Arrows point to the average length of phase in seconds in the adult in backward and forward pulsation, showing the abrupt shortening of the phases after eclosion.

Fig. 4 Average rate of pulsation, in seconds per phase, shortly before, during, and after pupation, as well as in the adult. The solid line gives forward, the broken line backward, beating.

Fig. 5 Average number of beats per phase in the larva approaching pupation, the pupa up to twelve to twenty-four hours, and the adult moth. The solid line represents forward, the broken line, backward beating; abscissas give age of the pupa in hours; ordinates, number of beats per phase.



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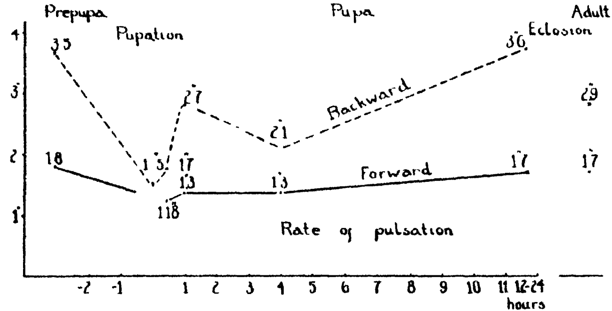

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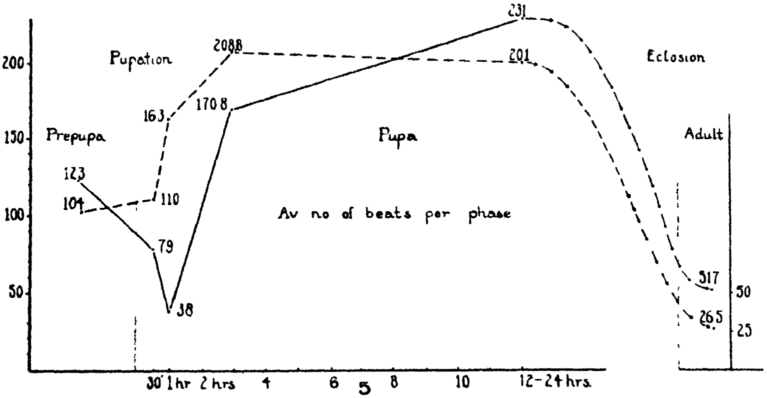

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Figures 3 to 5

changed very little in forward beating (1.3 seconds), but increased in inverse action to 2.76 seconds. That is, the inverse pulsation is now only about half as fast as at pupation; but, since its phases are long, the number of beats (average 163) in one of them is far greater than in the now temporarily diminutive forward phases.

One and one-half to four hours

During the period from one to four hours after pupation, the rate of pulsation, as seen by the interval between successive beats, changes very little in forward beating, but becomes slightly more rapid in backward peristalsis, the interval being reduced from 2.76 seconds to 2.10 seconds (fig. 4). Nor does the length of phase (fig. 3) change much in the inverse pulsation, though it increases strikingly in forward peristalsis. In both forward and backward beating the length of phases are now, after the fluctuations accompanying pupation have ceased, practically equal to those of corresponding phases before pupation, the present averages being 3 minutes 44 seconds forward, 7 minutes 13 seconds backward.

The most striking change that has come about is the enormous increase in the number of beats in a phase, especially in forward beating. Following pupation, when forward beating was temporarily discontinued, forward phases were exceedingly short and the number of beats in them correspondingly few. One hour after pupation, when backward beating was still dominant, forward beating was depressed, not in rate, but in length of phase and number of beats (average length, 50 seconds, 38 beats). Two hours later (3 hours after pupation), the average length of the forward phases and the average number of beats in a phase increased nearly four and one-half times (average length 3 minutes 44 seconds, 170.8 beats).

Meanwhile, the inverse pulsation has become slightly more rapid and with phases remaining of about the same length, the number of beats in a phase reaching a maximum for inverse beating in the pupa (208.8).

Twelve to twenty-four hours

The data obtained for the period twelve to twenty-four hours are sufficiently similar to be treated as a whole. Since the period two to four hours after pupation there has been a striking increase in the average length of the respective phases. In both inverse beating (12 minutes seven seconds vs. 7 minutes 13 seconds) and in forward pulsation (6 minutes 35 seconds vs. 3 minutes 44 seconds) it has nearly doubled (fig. 3). The individual beats have become infrequent, especially in the backward beating in which the interval between them (pulse rate) now averages 3.6 seconds (16.6 per minute). This slowness is connected with the falling off of the number of beats in a phase which in inverse pulsation now is less than in the forward (201 beats backward vs. 231 forward, fig. 5)—the reverse of the relation maintained since pupation, but similar to that observed before pupation.

A very remarkable feature characteristic of this stage in the life of the pupa is the occurrence of long pauses, usually in the midst of inverse phases. For instance, in individual no. 5, twenty-five to twenty-seven hours after pupation, after 373 inverse beats had occurred, a pause of 12 minutes 42 seconds ensued, after which 45 more beats in the same direction were noted before the reversal to beat forward. During the pause the vessel either shows no pulsation whatever or occasionally irregular faint undulations of the lateral walls are observed, one or two or three at a time, not recognizable as peristalsis in a definite direction and not at all like the full, strong pulsations which follow.

We notice here that pauses are frequently interpolated between parts of the backward phase. In the adult moth, on the other hand, they usually come at the end of a forward phase, before the reversal. In adult life they rarely, if ever, come at the end of a backward phase. After the slow beats of this phase fade out, quick forward pulsation appears directly, suddenly, and usually with full pulse.

Rate of pulsation during the pupal stage

The normal length of pupal life in the stock of Bombyx mori with which I worked was fourteen to twenty-one days. In 1928, I devoted especial attention to the rate of pulsation in pupae of advanced age, up to ten days. This seemed impossible during the earlier studies of 1927, on account of the increasing opacity of the cuticula after the first twenty-four hours, but I discovered that the difficulty of observing pulsation in the older pupae (without vivisection) was more apparent than real. The older pupae are subject to long pauses in peristaltic action which mislead the observer into thinking that nothing further is to be seen. But by choosing a favorable individual and waiting sometimes a half-hour or more for a pause to cease, one is rewarded by seeing with satisfactory clearness vigorous pulsations and periodic reversal.

An outstanding feature of the circulation in the pupa of eight to ten days is the quickening of the rate of backward peristalsis to that characteristic of the young adult.

Following the rates of forward and backward peristalsis in a single individual, 1928 no. p♀, from thirty-four hours before pupation to ten days after it, as shown in figure 6, one is struck with the evenness of the average rates in the phases of forward beating throughout all this long period. The prevailing average rate is one beat in 2 seconds, never slower than one in 2.2 seconds nor faster than one in 1.5 seconds.

Thirty-four hours before pupation, the average of three forward phases was one beat in 1.5 seconds; 9 hours and 2 to 4 hours before pupation the action was very slightly slower (one beat in 2 seconds). Meanwhile the characteristic speeding up of backward beating just before pupation was occurring (from one beat in 4.4 seconds to one in 3.3 seconds), reaching one beat in 1.8 seconds during pupation. Forward beating, as usual, did not occur during pupation. Soon (22 minutes) afterward, however, the average rate forward was 1.6 and the backward 2.2 seconds.

As shown by the graph (fig. 6), the average rate forward maintained practically the same level at 15, 25, 40, 60, 75, 86, 90 hours and at 5½, 8½, and 10 days. The fluctuations in the averages were too slight to be significant. The pulse of the oldest pupal stages observed (one in 1.9 seconds) is close to that of the young adult (1.7 seconds).

On the other hand, the average rate of backward beating was not only slow, but exceedingly variable: 15, 25, and 40 hours after pupation, the average was around one beat in 6.5 seconds, but the range of the variation between averages taken under similar conditions in closely recurring backward phases was between 8.4 seconds and 4.4 seconds for a single beat. These are probably the indices of rapid physiological changes accompanying metamorphosis. Possibly ampler observations might have shown similar variations after 40 hours, when the average observed was one beat in 6.3 seconds, but from that time onward the backward pulse quickened and simultaneously became more steady. The rate of one beat in 4.4 seconds observed at 72 to 74 hours was observed again at 86 hours and at 135 hours (5½ days). At 8½ days the backward pulse became still more steady and still more rapid, showing an average of one beat in 3 seconds, practically the same as that observed 2 days later and in the adult (2.9 seconds).

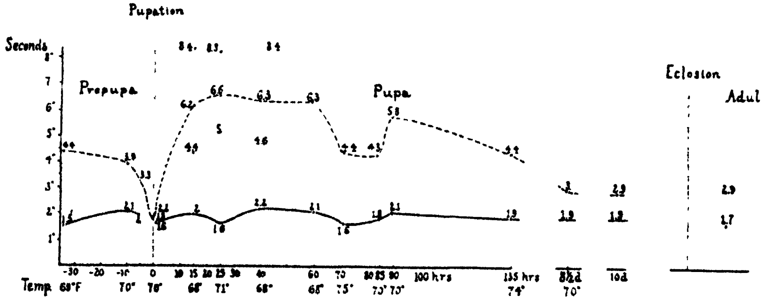

Fig. 6 Average rates of pulsation in a single individual (p♀, 1929), including advanced stages (to ten days) in the pupa.

Summarizing the observations of the average rates of the backward pulse in a single pupa at practically constant temperature, we see that, except at pupation, when it becomes equally rapid as in forward beating, the pulse during the first two or three days of pupal life is exceedingly slow and extraordinarily variable, after that more rapid and less variable. After the first week the average rate backward, instead of being roughly three times as slow as that forward, as was the case early in the first week, is only about one and a half times as slow—a relation which is carried over without change into the adult.

Changes in rate during single phases

The variations just described deal only with averages of whole phases. Either the entire time in seconds occupied in forward (or in backward) beating was divided by the number of beats, as in the observations of 1927, or series of ten beats taken as frequently as convenient throughout each whole phase were carefully timed and averaged, as in the more recent observations. The latter method disclosed the fact that there is generally a *very gradual and even retardation in the rate of beating during each single phase.* This is clearly shown in long phases, and can be accurately measured when the observer operates a stop-watch himself rather than calls off the changes to an assistant to be measured and recorded.

The only observed exception to this rule was the gradual acceleration in the phase of backward beating in connection with pupation, already described.

As examples of the characteristic retardation in the rate we may take two consecutive phases, one forward, one backward, in pupa 1928 p ♀ :

Forward: 10 beats in 15, 17, 15, 15½, 15⅕, 16⅖, 17⅘, 18⅖ seconds, followed by:
Backward: 10 beats in 39, 31⅕, 33, 35⅖, 39, 41⅕, 44⅕, 49⅖, 54⅕, 64, 69½, 74, 77 seconds.

Pauses

Complete interruption of peristalsis for periods lasting not infrequently over an hour is characteristic of the pupa. The longest pauses observed were from sixty to ninety hours after pupation. In individual 1928 no. p ♀ at 62 hours a pause of 57½ minutes occurred; at 72 hours a pause of 51 minutes occurred; at 86 hours a pause of 1 hour 24 minutes occurred; at 91 hours a pause of 1 hour 17½ minutes occurred. All of these pauses came at the end of forward phases and all were, so far as could be observed, complete interruptions of peristalsis.

Pauses sometimes occur in the midst of a backward phase, as occurred in this same pupa at 42 hours. After 60 beats backward in 276 seconds (= 4 minutes 36 seconds) a pause of 13 minutes 23 seconds ensued, followed by what may be called another installment of the same backward phase, viz., 157 beats in 960 seconds (= 15 minutes 59 seconds). The average rate in the first installment was one beat in 4.6 seconds, in the second much slower, one in 6.0 seconds—a variation characteristic of this stage in the life of the pupa.

A long period of inactivity broken only by faint pulsations occurred in 1928 no. p ♀ at 5½ days (134 hours). Coming at the end of a period of active forward pulsation lasting 13 minutes, it was observed for 1¼ hours. A few straggling faint strokes, either singly or in short groups, occurred, first backward, then forward, then backward. Such feeble action is exceptional and in marked contrast to typical peristalsis.

PERIODIC REVERSAL IN THE ADULT MOTH

Periodic reversal of peristalsis in Bombyx is characteristic of the adult as well as of the mature larva and the pupa. It may easily be observed at any time during the two or three weeks of adult life in the female or the slightly shorter span of life in the male.[5]

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[5] The nineteen female moths of which I secured data as to the duration of life in 1927 lived an average of 13 days, with a range from 4 to 22; fourteen males lived an average of 11.5 days, with a range from 4 to 18.

There are, however, certain peculiarities in the circulation of the adult as compared with the pupa immediately after pupation, which will now be mentioned.

Complete reversal. The moth shows complete antiperistalsis of the whole dorsal vessel in the inverse phase, unlike the young pupa, which in Bombyx exhibits 'double action,' that is, an inverse phase in which peristalsis starts between the third and fourth abdominal segments and runs in both directions, backward toward the caudal end of the heart and forward through the aorta into the thorax.[6]

Shorter phases. Another change characteristic of the moth is the shortening of the phases. The long phases observed in the pupae 12 to 24 hours after pupation, viz., 6½ minutes forward, 12 minutes inverse, on the average, with 231 beats forward, 201 inverse, are reduced in the young moth, as a rule, to 2 to 3 minutes or less, and usually consist of less than 100 beats, with notable exceptions to be mentioned (pp. 413, 414).

Senility. The onset of old age from five or six days onward abruptly diminishes still further the length of phase and number of beats in a phase. Single beats forward and backward, alternating with one another, often occur during and especially toward the end of this period.

Individual differences. Great irregularities peculiar to individuals, especially males, make it difficult to apply general rules to length of phase and number of beats in a phase.

Sex differences. Another adult characteristic is the appearance of marked sex differences in circulation. In general, the female shows an evener balance between the length of forward and inverse phases, less variation in the number of beats in a phase, than the male.

Mating usually tends in both sexes to increase the average number of beats in a phase and the average length of phases.

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[6] In the chrysalis of butterflies of the genus Colias 'double-action' from abdominal somites 3 to 4 occurs during the first part (about 12 beats) of the inverse phase and is followed by complete antiperistalsis during the rest of the phase, thus combining both the pupal and the imaginal types of reaction of Bombyx.

A notable exception to the last two conclusions drawn from the studies of 1927 was found in 1928 in the case of a female observed on the day of eclosion and before mating, in the midst of a long backward phase of more than an hour and more than 1500 beats. While this was continuing, she was mated. Normal short phases of less than 80 beats, alternating in direction, began immediately upon mating and were watched for over a half-hour.

Pauses and conflicts. Pauses much longer than those between the slow successive waves of antiperistalsis, though not as long as in the older pupae, often occur, usually at the end of a forward phase. Waves of peristalsis originating in the thorax sometimes conflict for a few strokes with those from the posterior end of the abdomen. They meet each other in the middle or front of the abdomen. Finally, one phase, usually the forward, gets the upper hand and continues, while the opposing waves cease altogether.

The twelve- to twenty-four-hour pupa. To go back to the end of the first day of pupal life, the alternating backward and forward phases have lengthened and are frequently broken by long pauses. My notes show an average length of 12 minutes 7 seconds of backward, 6 minutes 35 seconds of forward, phases. Unbroken backward phases over 22 and 24 minutes long were observed, and pauses up to 12 and 18 minutes long were interpolated among the parts of a broken backward phase. The average number of beats in a backward phase, owing to the slower rate, is now less than in a forward phase (201 backward, 231 forward). The average rate (seconds from beat to beat) was 1.7 seconds forward, 3.6 seconds backward.

These data of the twelve- to twenty-four-hour pupa may now be compared with those obtained from five young non-mating moths, three females and two males, during the first four days after eclosion.

TABLE 4

Young, non-mating moths

			FORWARD			BACKWARD		
Age	Sex	Individual	Average number of beats	Average length of phase	Rate	Number of beats	Average length of phase	Rate
				Seconds	*Seconds*		*Seconds*	*Seconds*
Freshly emerged	♀	7	36	47	1.3	29.8	71	2.37
1 day	♀	a	78	138	1.77	43.2	125	2.89
2 days	♀	a	55	103.5	1.87	39	122.7	3.15
3 days	♀	a	141	214	1.5	48.5	116	2.39
4 days	♀	2	55.3	95.3	1.7	8.2	21.3	2.6
1 day	♂	b	21.3	39.7	1.86	19	59	3.06
2 days	♂	b	17	29	1.69	11.7	38	3.29
4 days	♂	b	16	52.6	3.24	12.5	56.3	4.5
3 days	♂	d	45.9	47.7	1.04	27.2	52	1.9
	Averages		51.7	63	1.77	26.5	73.47	2.9

This table brings out the great reduction in the number of beats in a phase and in the length of the phases since the 12-to-24-hour pupal stage, in both forward and backward beating. The length of phase, whether forward or backward, is roughly one or two minutes (average 63 seconds forward and 73½ seconds backward), instead of 6½ and 12 minutes. The number of beats in a phase is now less than 100 (average 51.7 forward, 26.5 backward) as compared with over 200 forward and nearly 200 backward in the pupa.

It is an interesting fact that the predominance of the forward phase, as to the number of beats, acquired during the pupal life six or eight hours after pupation (fig. 5), still is maintained. During pupation backward beating held sway exclusively and predominated in the average number of beats per phase for several hours thereafter. Mating and internal conditions connected with senility modify it, but, as the table shows, the average number of forward beats in these young unmated moths in every case exceeds the backward.

The rates of beating have not essentially changed since the 12-to-24-hour pupal stage, though backward pulsation is slightly more rapid now than then (2.9 seconds now, 3.6 seconds in the pupa), but forward pulsation (average 1.7 seconds) remains more rapid than backward.

TABLE 5

Effect of mating on the female

		FORWARD				BACKWARD		
	Age	Individual	Beats, average	Average length of phase	Rate	Average beats	Average length of phase	Rate
	Days			*Seconds*	*Seconds*		*Seconds*	*Seconds*
Before	4	2	55.3	95.3	1.7	8.2	21.3	2.6
During	4	2	106.3	191.0	1.8	18	57	3.17
Before	0	7	36.2	47	1.3	29.8	71	2.37
During	0	7	126	120.3	0.95	21	67.3	3 18
Before	6	a	1.3	9	6.8	1.4	7.6	5.33
During	6	a	Av. 16 1 of 145	Av. 47.8	2.3	28	108.2	3.8
				On the male				
Before	2	b	17	29	1.69	11.9	38.5	3.29
During	2	b	35.7	83	2.32	315	545	1.73
Before	4	b	16.2	52.6	3.24	12.5	56.3	4.5
During	4	b	35	144	4.1	275	500	1.82
Before	3	d	45.9	47.7	1.04	27.2	52	1.9
During	3	d	77.2	92.2	1.19	65.6	119.6	1.8
Before	6	3	9	18.8	2.08	9.9	27	2.75
During	6	3	1.3	2.7	2.07	29.3	59	2.00

The effect of mating on periodic reversal

Silkworm moths mate on the day of eclosion, the instinct for pairing being so strong that any male and female couple almost instantly when placed in the same vicinity. The female is nearly stationary, while the male before mating flutters about her within a radius of a few inches. Smell dominates, sight seems to play no part in mating.

To keep a very active male quiet during observation of the pulse, it is convenient to allow him to mate. Then the scales can readily be rubbed off from his back and observations made on the dorsal vessel.

Does mating affect the phenomena of periodic reversal of peristalsis, and, if so, in what particular way? To answer these questions I made, in 1927, consecutive observations before and during mating on three females and three males. One of the latter was observed in this way on August 12th and again on August 14th. This gave a total of fourteen tests on forward, fourteen on backward peristalsis shown in table 5, p. 411).

General conclusions regarding the effects of mating

Mating in both sexes tends to increase the average number of beats in a phase and average length of the phases. This applies both to forward and to backward pulsation. In twenty-eight pairs of averages this result holds for all but four. These exceptions are the case of backward pulsation in female 7, in which a very slight decrease occurred, and forward beating in an old male, 3 (six days after eclosion), the phases of which consisted almost altogether of single beats.

The rate of forward pulsation was apparently accelerated by mating in two of the three females tested (unchanged in the third), retarded (in one case unchanged) in the males.

The velocity of backward pulsation was apparently retarded in two of the three females, accelerated in the third, a, six days old, with abnormally slow pulse before mating, accelerated in males (three tests), unchanged in one male.

Mating in the male, accordingly, judging by the limited data at hand, accelerates backward peristalsis to a rate (1.8 seconds) equal to what may be regarded as the normal forward rate, whereas the rate of forward pulsation in the same observations is retarded in a very marked degree. The dominant stimulus controlling the pulse of the male during mating evidently acts from the head.

Long phases in the male

The prodigiously long backward phases in male b during mating tend to support this view. Tests made on him at the ages of two and four days both gave similar results. Unmated at two days, his pulse backward was abnormally slow and the phases short, averaging 11.7 beats in 38.5 seconds. During the immediately subsequent mating, a long backward phase of 20 minutes 10 seconds with 719 beats occurred. The next backward phases were shortened to 3½ minutes, but they were longer than any backward phases of the preliminary observations for control.

At the age of four days his pulse backward was quickened from a rate of one beat in 4.5 seconds to one in 1.8 seconds and an extraordinarily long backward phase (275 beats in 8 minutes 21 seconds) was again observed. These backward pulsations were recorded as "vigorous full beats, becoming weak at the 225th."

Extraordinarily long phases in the male, however, are not necessarily backward. The first male which came under my observation showed both when mating and when free some very long forward phases. Unfortunately, no consecutive control observations were made in connection with these data, so that one cannot say what affect, if any, mating had in the production of these long phases, but the tendency of this individual toward long forward phases was very striking. One forward phase lasted for over a half-hour (32 minutes 26 seconds) with 1281 beats, another for 7 minutes 27 seconds with 255 beats, as contrasted with backward phases of less than one minute (59 seconds, 52 seconds, 26 seconds) and correspondingly few beats (26, 24, 10). These observations were made on the day following pupation, during mating. The next day, when remated with the same female, he gave normal data as regards length of phase as compared with observations on other males and especially females.

But the following day (three days after eclosion), when not mated, he repeated the same performance as on the second day previous, a short backward phase of 4 beats in 12 seconds, followed by a long forward phase of 19 minutes 38 seconds with 750 beats, then another short backward.

This individual was interesting as showing the remarkable variability of males in periodic reversal.

An exceptional female. A female in 1928 broke every rule tentatively drawn from the data of 1927. On the day of eclosion, before mating, she was observed in a long backward phase of more than an hour (1 hour 10 minutes +). During this time there were 1545 pulsations in 54¾ minutes, a rate of 2.1 seconds. Such a long backward phase had never been seen in an adult female. While this backward phase was still in action, a mating was brought about. Immediately normal periodic reversal began, which was followed for over a half-hour. Four consecutive phases at the beginning were typical:

Forword	*Backward*
48 beats, 60 seconds, rate 1.25 seconds→	26 beats, 48 seconds, rate 1.8 seconds
76 beats, 96 seconds, rate 1.26 seconds→	19 beats, 36 seconds, rate 1.8 seconds

The average rates during mating were 1.5 seconds forward, 1.9 seconds backward. No phase during the next half-hour exceeded 80 beats. Thus, short phases, a slightly quicker rate backward, and a still faster rate forward, were the immediate result of the mating, and every conclusion previously drawn in regard to the effect of mating was contradicted. An abnormal lack of balance was replaced by a normal equipoise between forward and backward peristalsis.

Variations accompanying old age

The study of two females and one male at different stages of adult life suggests the possibility that certain variations in the phenomena of circulation may be expected to occur toward the end of life. The data are too meager to be conclusive and do not in themselves indicate that these variations are bound to occur in every individual, but only that they do sometimes occur and may be expected at an advanced age.

Female a, on the morning of August 15th, six days after eclosion and two days before her death, gave the following averages:

Forward			Backward		
Beats	Length of phase, seconds	Rate, seconds	Beats	Length of phase, seconds	Rate, seconds
32	46	1.4	54.6	131	2.4

She was still fairly normal, giving results similar to the readings of early adult life (1, 2, 3 days old; table 4) except that the average numbers of forward beats and of length of phase are lower than the averages for backward pulsation.

Two readings taken on the afternoon of the same day (at 2.47 and 4.12 P.M.) gave a strangely different result:

Forward			Backward		
Beats	Length of phase, seconds	Rate, seconds	Beats	Length of phase, seconds	Rate, seconds
1.7	10	6.3	5.7	4.6	8
1.3	9	6.8	1.4	7.6	5.33

An abrupt change has come about, viz., the reduction of phases of normal length characteristic of earlier life to very short phases or to single pulsations, with such pauses between them as to lower the rate forward to one beat in 6.3 seconds or 6.8 seconds, whereas in the forenoon of the same day the average length of a beat was 1.4 seconds. Similarly in the backward phases, the rate changed from one beat in 2.4 seconds to one in 8 seconds and 5.33 seconds.

Another female, x, which lived to be eighteen days old, gave the following normal averages at the age of nine days:

Forward			Backward		
Beats	Length of phase, seconds	Rate, seconds	Beats	Length of phase, seconds	Rate, seconds
66.5	148	2.27	65.3	188	2.87

At fifteen days the averages were:

Forward			Backward		
Beats	Length of phase, seconds	Rate, seconds	Beats	Length of phase, seconds	Rate, seconds
39.6	70	1.76	28	227.6	8.13

Great changes have come about in backward pulsation which are only partly indicated by these averages, which show, however, the slowness of pulsation in the three observed backward phases, viz., 1 beat in 8.13 seconds as compared with 1 in 2.87 seconds. Ten of the forward phases counted (ranging from 4 beats in 10 seconds to 142 beats in 3 minutes

32 seconds) were followed by pauses (ranging from 5 seconds to 90 seconds) with no subsequent backward phase, no reversal. The extremely slow rate was directly associated with the failure, during the major part of the period of observation, to reverse.

At sixteen days, in observations made both in the morning and the afternoon, phases reduced to a single beat frequently appeared among the phases of both forward and backward pulsation. The rate of backward beating remained inordinately slow (1 beat in 6.6 seconds and 9.3 seconds).

At seventeen days the phases were all short, but none were reduced to single beats. The rate of backward beating, however, was still extraordinarily slow, as it had been since the age of fifteen days.

Forward			*Backward*		
Beats	Length of phase, seconds	Rate, seconds	Beats	Length of phase, seconds	Rate, seconds
27	32.9	1.2	11.8	64.0	6.29

A male, 4, which lived to be eleven to twelve days old, gave, when ten days old, the following record of averages:

Forward			*Backward*		
Beats	Length of phase, seconds	Rate, seconds	Beats	Length of phase, seconds	Rate, seconds
85	110	1.29	93	149.2	1.6

The next day, however, when nearly dead, the averages, including eight single beats (each representing one phase in the periodic reversal), were:

Forward		
Beats	Length of phase, seconds	Rate, seconds
7.9	18.6	2.3

and, including two single beats,

Backward		
Beats	Length of phase, seconds	Rate, seconds
5.4	10.2	1.88

In brief, he showed the reduction of the length of phase, and, especially, the limitation of phases to a single beat, without the slowing up of the rate of backward pulsation observed in the two females. The rate forward, however, was slightly slower.

Temperature

The observations recorded in this paper were made under approximately constant external conditions of temperature and light, during August (July 26th to September 2nd), in a brightly illuminated room with southern exposure, though the insects were not exposed to direct sunlight. The moths were kept, when not being observed, in a slightly cooler, less well-lighted room. The temperature at which the observations were taken varied comparatively little (21° to 25°C.). No systematic study of the effects of different temperatures on the rate of pulsation was made. This has been already done for the larval silkworm with precision by Crozier and Federighi ('25).

A few observations were taken, however, to determine what effects, if any, high and low temperatures would have upon periodic reversal in the adult. They were made upon ♀ (x) placed in a large test-tube containing a thermometer held in position by a cork stopper. The tube was placed in a water-bath kept at a temperature as nearly constant as possible by an electric heater. The moth had been exposed to the high temperature about twenty-five minutes before the readings were taken. The fall of 2¼°C. during the eleven and one-half minutes of the observation had no observable effect upon the rate in earlier and later phases. The control observations at normal temperature (22°C.) were begun thirteen minutes after the end of the first experiment, when the moth had become accustomed to the lower, normal temperature. All readings were taken with a Leitz binocular magnifier.

Temperature, 37° to 34.5°C.

	Forward			*Backward*	
Beats	Length of phase, seconds	Rate, seconds	Beats	Length of phase, seconds	Rate, seconds
			46	80	1.7
91	80	0.88	27	50	1.85
84	75	0.89	24	40	1.66
75	70	0.93	28	44	1.57
69	65	0.94	55	90	1.63
80	70	0.87	19	26	1.37

Temperature, 22°C.

	Forward			*Backward*	
Beats	Length of phase, seconds	Rate, seconds	Beats	Length of phase, seconds	Rate, seconds
			112	310	2.76
57	116	2.0	48	146	3.04
76	180	2.36	36	108	3.00

Summary

Temperature, 37° to 34.5°C.

Beats	Length of phase, seconds	Rate, seconds	Beats	Length of phase, seconds	Rate, seconds
79.8	90	0.90	33.1	55	1.65

Temperature, 22°C.

66	148	2.22	65	188	2.87

This single set of observations indicates that the number of beats in a phase is probably not affected by the higher temperature, but that the average length of phase in seconds and the average interval between single beats are very much diminished by the rise in temperature in both forward and backward pulsation. In other words, acceleration of the pulse is accompanied by a shortening of the phases.

Low temperatures. The following day a few observations were made with the same female at low temperatures (8° and 10°C.), following them with observations at normal (21°C.).

The large test-tube containing the moth and a thermometer was placed in a basin of water containing ice. After thirteen minutes of exposure to 8°C., the rate of beating forward was 1 in 5 seconds; backward, was 1 in 12 seconds. At 10°C. the rate forward was 1 in 4.9 seconds; backward, was 1 in 13.5 seconds.

(Thus the interval between two successive backward beats was not reduced by the 2° rise in temperature, but was slightly lengthened.)

The control reading gave us the average of five forward and two backward phases at temperature 21°C.; the rate: forward, 1 beat in 2.09 seconds; backward, 1 beat in 4.6; 5.9 (if all five phases are included, some with long pauses).

Summary. Rate, i.e., length from one beat to the next:

	Forward, seconds	*Backward, seconds*
At 21°,	2.09	4.6
At 10°,	4.9	13.5
At 8°,	5.0	12

The phases at low temperatures are very long, but no exact records were made of them.

EXPLANATION OF PERIODIC REVERSAL

Similarities between periodic reversal in Lepidoptera and Ascidia

In spite of the striking differences between the circulatory systems in these two groups, there are remarkable resemblances between them in physiological reactions and to a certain extent in morphology. The ascidian heart (Hecht, '18) is a simple muscular tube, ventral in position, lying within a somewhat larger tube, the pericardium. It communicates anteriorly with the hypobranchial vessel, supplying the branchial walls, posteriorly with the visceral vessel. At each extremity the blood vessels end in sinuses.

Advisceral peristalsis, alternating with abvisceral, is the more rapid, with two or three times as many beats in a phase, pumping aerated blood from the branchial region into the viscera.

Physiologically, advisceral circulation corresponds in force and rate with forward peristalsis in Lepidoptera, for in the latter the rate is also more rapid than in backward, or inverse, peristalsis.

The 'central beat' described by Hecht, "originating in the region between the two ends that normally originate the beat," in which two waves "proceed simultaneously, one in the advisceral and the other in the abvisceral direction," has its counterpart in that which I have called 'double action' and which Bataillon ('93) designated as 'circulation indifférente' in the Lepidoptera. The similarity, however, will not stand close inspection, for there is no evidence, so far as is known, that the ascidian heart has lateral ostia or that the 'central beat' has any 'central' source from which to draw blood; consequently, this phenomenon in ascidians is of no such functional importance as in the larva and pupa of the silkworm in which 'double action' is nearly or quite as common as complete antiperistalsis. I have seen conspicuous streaming into the pericardium in abdominal segments 3 to 4 of blood from the ventral sinus made visible by injection of India ink. This is the source of supply for the central beat in the silkworm.

It is certain that there is a real circulation in the 'central beat' of Lepidoptera. If one is skeptical on this point, he has only to observe a larva of Colias or of Bombyx in the act of pupation to see prolonged and vigorous double action which involves unmistakable circulation.

Hecht maintains that in the three species of tunicates which he studied there is a definite one-way circulation of the blood, in spite of periodic reversal of peristalsis, and Fischer ('18), following the long-standing idea that the heart is so equipped with valves that the blood cannot flow backward, made a similar suggestion for Lepidoptera. However this may be for Ascidia, on which I have never made observations, it is certainly not true of the silkworm and of butterflies of the genus Colias, as the following experiment shows. I injected into the ventral sinus of an adult Bombyx an emulsion of Chinese ink in tap-water, after rubbing off the scales of the back and one side. Waves of black fluid in the dorsal vessel swept backward to the end of the body, each sharply distinguished against the uncolored portion of the heart just behind it. Then came reversal with pulsation forward, and the pericardial and lateral sinuses became full of black fluid. Eventually, after two hours or more of beating forward and backward, the contents of the dorsal vessel became colorless, being diluted with fresh blood, and the black pigment having become scattered through the sinuses. There was no doubt in my mind about the transfer backward of the black fluid in antiperistalsis.

Fischer's ('18) quandary as to how blood could circulate backward against valves and into a cul de sac is easily answered. There are no valves to obstruct the lumen of the tube, and during the slow inverse pulsation the posterior end of the heart presumably is relaxed and the ostia wide open. Contraction of this end in forward peristalsis, on the other hand, naturally closes the ostia and their valves, so that blood cannot escape and is propelled forward.

Theories of the cause of periodic reversal

The suggestion by Fischer that antiperistalsis may mean an acid condition of the blood was taken from human pathology and based on the idea that periodic reversal is a transitory disturbance accompanying metamorphosis. He was evidently unaware that Bataillon ('94) had advanced a somewhat similar theory, viz., that the accumulation of CO_2 in the blood of the silkworm accompanying histolysis of the tissues on the day preceding and the very day of pupation is responsible for periodic slackening in circulatory rhythm. Pupation therefore, according to Bataillon, is accompanied by temporary asphyxia.

[Bataillon ('94), in a paper which has very recently come to my attention, describes periodic reversal in the adult moth, thus reversing his opinion that it is a transitory phenomenon. Recent experiments by Yokoyama ('27) confirm the idea that acidity induces periodic reversal. These facts will be brought out in a paper now ready for the press.]

In ascidians the blood has an acid reaction; Hecht has found that the acidity is resident in the corpuscles, not in the plasma. No suggestion has come to my attention that acidity of the blood in this group has anything to do with periodic reversal.

In my earlier papers on this subject ('24 a, b) I called attention to the fact that periodic reversal comes on in those days or hours when beneath the caterpillar's skin the wasp-waisted form of the butterfly first appears, and suggested the theory that periodic reversal is the result of the valve-like action of the constricted base of the abdomen which probably restricts the flow of blood through the sinuses and increases in blood pressure alternately, first in the thorax and then in the abdomen. My opinion was supported by the fact that the parasitic copepod Lepeophtheirus pectoralis described by Scott ('01), with a very narrow waist, has periodic reversal of lacunar circulation between the cephalothorax and the 'genital segment.' There being no heart, the blood in Lepeophtheirus is propelled "by successive jerks, depending upon the movements of the alimentary canal and, in part, of the reproductive system."

A corollary of this theory was that the narrow waist, blocked by cords of longitudinal muscle, serves as a valve during the filling of the wings with blood at eclosion. Hasbrouck ('24), however, has recently advanced the idea that no blood pressure is necessary to fill the bag-like wing buds, that the capillary action of fluids between two parallel walls so close together as those are of the wing bud would be quite sufficient to fill and distend them without any pumping action from the abdomen or any increased blood pressure. While capillarity may be sufficient in a small moth, such as Cymatophora or, with which he was chiefly occupied, observations on larger Lepidoptera make it evident that the pressure of the muscular walls of the body upon the haemolymph plays a large part in filling the wing buds.

The back-pressure theory as to the cause of periodic reversal is much weakened by the fact, which I have recently ascertained, that when a median-dorsal strip of body wall of the silkworm moth with dorsal vessel attached is cut off from the body, inverted, pinned to a piece of cork, and exposed to moist air, periodic reversal will continue for hours, though its details may be quite extraordinary. In one case (August 24th) such a preparation (including the metathorax) continued to show periodic reversal for several minutes, even though there was no connection with the pulsating vesicle of the mesothorax, and then beat only forward for at least an hour. It was beating when I left the laboratory at 5.30

P.M. At 9 A.M. the following day (sixteen and one-half hours since it was prepared), it was beating backward.

Malpighi's observations of reversal in the two parts of the severed heart are perfectly credible.

The back-pressure theory of the origin of periodic reversal thus receives no support from experiments with the excised heart, either in the silkworm moth or in tunicates, and yet why should hourglass-shaped organisms, like Lepeophtheirus and the caterpillar just as it becomes 'wasp-waisted,' show it unless this peculiar form has something to do with bringing it on? At least it seems worth while to search among other wasp-waisted insects than Lepidoptera and among other Crustacea for examples of it.

If alternating increase in blood pressure at the two ends of the heart do not bring about periodic reversal, what does? Two theories have been advanced in regard to the similar phenomenon in Ascidia, viz.: 1) the one advanced by J. Loeb ('02), that the opposing waves are of neurogenic origin, arising from ganglia at the two extremities having 'alternating dominance as pacemakers.'

This formal 'explanation' of the mechanism of periodic reversal implies that the mechanism is automatic, independent of the central nervous system; that first one ganglion, then the other originates waves of peristalsis. It tells us nothing of the temporary chemical or physical changes which cause the momentary dominance of one ganglion or the other. The terminal position of the ganglia is the important matter.

The other theory, 2) claiming the myogenic origin of the opposing pulsations, implies that "all the muscle cells are capable of an automatic rhythm" (Hecht, '18). Any portion of the heart may initiate pulsations.

In the normal Ascidian heart the two ends alternately act as pace-makers, due to the alternating superiority of their pulse rates. The balance in their favor over the central portion of the heart is, however, so delicate that it requires only a slight change of metabolic activity of the heart to upset it. The branchial end is normally the most potent part of the heart, as is evidenced by the much greater duration and rate of its activity.

The main argument advanced by Hecht ('18, pp. 180–181) for the myogenic origin of the beat, which thus rules out the necessity of nervous control, is that central beating "may originate at practically any place between the two ends."

Hunter ('02) found small ganglia in Molgula only "at the opposite ends of the heart where the waves of contraction originate," and found no evidence in this form of spontaneous central beating.

This seems to show that the neurogenic theory applies well to Molgula, though in other genera Schultze ('01) and Bancroft and Esterly ('03), as Hecht states, "found no difficulty in securing an automatic rhymicity of the isolated central part of the heart in ordinary sea water."

These theories, which differ essentially only as to whether terminal ganglia are present or not, may be applied to periodic reversal in Bombyx, as the following facts show: 1) The heart beats and reverses periodically when removed from the body attached to the dorsal wall; 2) in one individual in which the posterior end was discolored and evidently moribund, long-continued backward beating was observed, that is, the anterior end only was in condition to originate pulsations; 3) during pupation the distended heart in Bombyx beats only backward. In one male no aortic pulsation forward took place while the skin was being shed, and for a half-hour afterward there was no forward phase; 4) in old moths single beats occasionally start at the same moment from the two ends and conflict with each other near the middle of the heart; 5) not uncommonly in old moths single beats in succession, one forward, one backward, alternate with each other for a short time. These facts are inconsistent with the theory that periodic reversal is brought about by the stimulus of back pressure from the sinuses of thorax and head.

Similarly, the extraordinarily long backward phases of 20 minutes 10 seconds, 8 minutes 21 seconds, with 719 and 275 beats, respectively, observed on two occasions in ♂ b when mating, cannot be ascribed to the sudden release of accumulated blood in the thorax. It would not take twenty minutes to relieve such pressure.

We have here problems more fundamental than the back-pressure theory or than the neurogenic or myogenic origin of the beat. What are the factors underlying differential muscular 'rhymicity' or ganglionic 'potency'? Account must be taken of the sources and chemical composition of the blood which at a given moment is streaming at various points into the pericardium from the sinuses. Presumably, if a large amount of blood is entering abdominal segments 3 to 4, as at the moment of pupation, 'central beating' is started, and the flow backward continues until reversal occurs, but what stimulates ganglia or muscle at the posterior end to start the forward wave?

SUMMARY AND CONCLUSIONS

1. Malpighi's discovery of periodic reversal of heart beat in the silkworm moth and pupa has remained almost in oblivion for 260 years. Bataillon ('93) described periodic reversal in the prepupa and pupa as a transitory phenomenon due to asphyxiation, holding that peristalsis in the adult is forward, as in the larva. Fischer ('18) and Gerould ('24 a, b) independently rediscovered periodic reversal in various Lepidoptera, especially Colias.
2. The dorsal vessel of the silkworm forty-eight hours prior to pupation no longer beats constantly forward, but phases of rapid forward beating alternate with phases of slower backward peristalsis.
3. Pupation occurs during a vigorous backward phase, wholly backward or, in some individuals, backward through the abdomen from the fourth abdominal segment, forward through the aorta from the third (double-action, diverging waves).
4. Soon after pupation, backward phases for a few hours are of the latter type. Later, and in the adult, complete reversal regularly occurs.
5. In the early hours of pupal life, the average length of both backward and forward phases increases, but the backward phases average about twice as long as the forward (reaching 15 minutes vs. 6 minutes 30 seconds).

6. The average number of beats in a phase, meanwhile, rapidly increases, and the backward phases temporarily exceed in this respect the forward, until the slowing down of the backward pulse at eight to ten hours brings the average number per phase backward below that of forward beating.

7. The average pulse rate of forward beating in the prepupa, pupa, and adult remains nearly constant at constant temperature. It is roughly twice as fast as in forward beating.

8. The average pulse rate of backward beating, however, increases rapidly just before pupation, until at pupation it equals that of forward beating; fifteen to forty-eight hours after pupation, it is extraordinarily variable and slow.

9. The backward pulse rate in the pupa of eight to ten days becomes faster than in the younger pupa, and the same rate is maintained in the adult.

10. Any long phase, whether backward or forward (except the backward when approaching pupation), shows a gradual retardation in pulse rate.

11. Pauses (sometimes one and one-half hours long in older pupae) often follow forward phases.

12. In the adult moth forward and backward phases, as a rule, become enormously shortened, to about one minute, the backward phases averaging slightly longer than the forward.

13. Owing to the slower average pulse rate of backward as compared with forward beating, however, the opposite phases being not very different in length, the average number of beats per phase is less in backward than in forward beating, as was the case in the prepupa.

14. The average backward and forward pulse rates remain the same, however, as in the eight-to-ten-day pupa, i.e., backward beating is slower (2.9 seconds per beat = 20.7 beats per minute); forward, more rapid (1.7 seconds per beat = 35.3 beats per minute).

15. Old age abruptly diminishes still further the length of phase and number of beats in a phase. Reduction to single beats often occurs.

16. Mating tends in both sexes to increase the average number of beats in a phase and average length of phase. Mating in the male accelerated backward, retarded forward peristalsis; it seems to have had exactly the opposite effect on the female, though the data are too meager to prove it.

17. Usually the female has shown an evener balance between the length of forward and backward phases and less variation in the number of beats in a phase than the male. In one case, however, an extraordinarily long backward phase was observed in a female. Mating interrupted the phase and restored normal equipoise between forward and backward peristalsis.

18. Certain males have a marked tendency toward exceedingly long phases, forward in certain individuals, backward in others.

19. Conflicting waves starting simultaneously at each extremity sometimes occur, until one phase, usually the forward, gets the upper hand.

20. The rate of backward beating was retarded more than forward peristalsis in lowering the temperature from 21°C. to 8° or 10°C., but only one trial was made.

21. Advisceral (backward) peristalsis in ascidians corresponds in force and rate to forward peristalsis in the silkworm, abvisceral (forward toward the branchial walls) to backward peristalsis.

22. Injections of India ink into the lacunae of the moth prove that antiperistalsis produces backward flow. Observations on the full pulse during the long backward phase, including pupation (lasting occasionally more than an hour), confirm this conclusion.

23. During backward peristalsis the ostia at the posterior extremity of the heart in all probability are open.

24. The constricted waist of the prepupa, pupa, and adult may play an important rôle as a valve in lacunar circulation, especially at crises like pupation and eclosion.

25. But it is improbable that back pressure of blood in the thorax is the cause of reversal to beat backward, because

the excised heart isolated upon a median-dorsal strip of body wall continues for several hours to beat backward and forward alternately. The alternation of single beats forward and backward frequently seen in old moths is also inconsistent with the back-pressure hypothesis.

26. The neurogenic 'pacemaker' theory of periodic reversal affords only a formal explanation of the phenomenon in Ascidia and Lepidoptera.

27. Malpighi's conception of the heart of the silkworm moth as a series of separate chambers is incorrect. The heart of the silkworm at all stages is a simple tube without internal valves.

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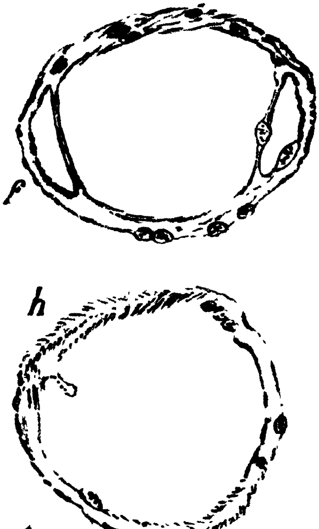
PLATE 1

EXPLANATION OF FIGURES

1 Reproduction of a part of Verson's ('08) Tav. II, fig. 29. Cross-sections of the dorsal vessel in the region of the first pair of ostia between thorax and abdomen in a chrysalis of Bombyx of the sixth day.

2 Reproduction of Verson's ('08) Tav. II, fig. 18. Frontal section through the dorsal vessel, showing the last caudal pair of ostia (*ost.*) in a silkworm larva of the third instar.



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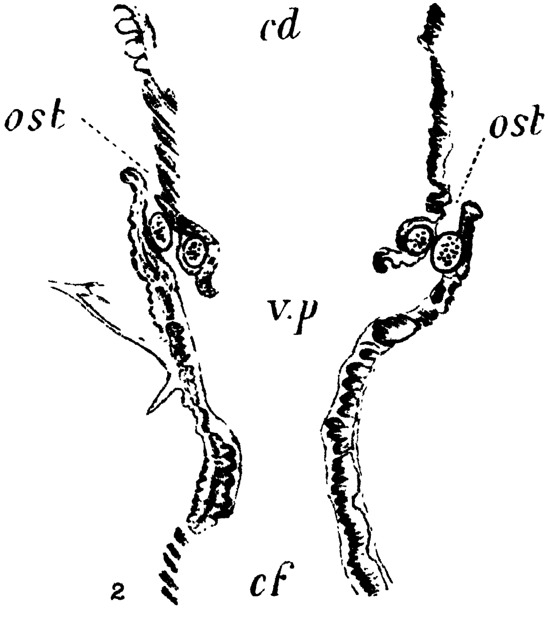

2

MATURATION OF THE MALE GERM CELLS IN THE RAT[1]

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TWO PLATES (TWENTY FIGURES)

AUTHOR'S ABSTRACT

Maturation of the male germ cells in the rat shows some slight modifications of the typical procedure in sex cells, a distinct and rather prolonged synapsis occurring before synizesis and a confused stage immediately after it. The clumping in synizesis is not extreme. In the mixed strain of rats both twenty-one and thirty-one tetrads appear in the late diakinesis. This procedure in the male resembles the maturation of the oocytes in only two points, the beginning of the process, the deutobroch nuclei, and the end of it when the haploid number of chromosomes take their places on the spindle.

Since it has been found that the process of oogenesis in the rat (Swezy, '29) does not conform to what has been considered the typical pattern, in that the usual maturation phases are omitted in the development of the definitive germ cells, it seemed necessary to study these phases in the male germ cells to determine whether this was a species character or a sex character. Duesberg's ('08) work on the white rat would lead one to suspect that it was a species character, as he did not find synizesis and synapsis in his material. However, the study of testes from both the white rat and our mixed strain of rats for other phases of chromosomal development did not reveal any striking lack in the maturation phases commonly seen in the testis, hence it was felt that Duesberg had not told the entire story. A careful search of this material was made, using both the white rat and our mixed strain of rats, and this amply confirmed our first impression that all the usual maturation phases occur in the development of the male germ cells, though slightly modified

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[1] Aided by grants from the Committee for Research in Problems of Sex of the National Research Council, administered by Prof. Herbert M. Evans, to whom the author is greatly indebted.

in some of the early phases, as will be pointed out below. These modifications, however, are not sufficiently great to justify their elimination as distinct phases.

The material used in this study was the same as that used in previous studies on the rat, the fixation and preparation of which have been given in earlier papers. This material has proved to be especially good for chromosome studies, both the primary and secondary spermatocytes, as well as the earlier phases, presenting none of the difficulties of analysis heretofore supposed to be inherent in mammalian chromosomes. There can be little doubt that this is wholly due to the speed in the initial steps in fixation (Evans and Swezy, '29) as well as the length of time taken for passing it through the various grades of alcohol. In general, it has been found that even well-fixed material may be spoiled for accurate chromosome differentiation by a prolonged bath in alcohol, hence the necessity of shortening this part of the preparation as much as may be consistent with good results.

The seriation of stages is fairly easy to follow, cells showing the early maturation changes, up to and including synizesis, being found only in the outer one or two rows of cells lining the tubules. The growth period which begins at the end of synizesis also aids in distinguishing the stages that follow. The diakinesis and later phases have invariably moved into the center of the tubule. The Sertoli cells may be distinguished from other cells by the elongate nucleus and the dark, more or less curved rod which extends the length of the cell, resembling in this respect the Sertoli cells in the human testis (Evans and Swezy, '29).

The spermatogonia appear as a single row of small, rather darkly staining, cuboidal cells immediately beneath the thin epithelial membrane, which is here only one cell in thickness. They take a darker stain and are smaller than the cells in the interior of the tubules. Next to this row of cells the primary spermatocytes are to be found. They are easily differentiated from the spermatogonia, as in most cases they show the beginning of the maturation changes in the nuclei.

In some sections the primary spermatocytes extend out to the epithelial membrane with no row of spermatogonia intervening.

The nucleus of the spermatogonium is relatively large and is filled with a fine linin reticulum with rather large masses or blocks of chromatin scattered through it (fig. 1). Other blocks of chromatin are found closely adherent to the nuclear membrane, which is rather dark and thick. It is difficult to decide which of the blocks of chromatin represent nucleoli, though there is usually one rather larger than the others, but with no other distinguishing mark.

The interval between the last spermatogonial division and the appearance of the earliest maturation changes in the nucleus of the primary spermatocytes is apparently a short one. The earliest change is shown in the breaking up of the chromatin masses into fine granular threads (fig. 2). These leptotene threads eventually become more definite in outline and completely fill the nucleus with a quite even regularity (fig. 3). Two or sometimes more nucleoli are present, one somewhat larger than the others.

Following the appearance of the leptotene threads, synapsis occurs. During this phase the threads may sometimes appear as two slender ones lying side by side, or in other cases only the ends are split, the union not yet being complete (figs. 4 and 5). The number of threads becomes very materially reduced, while at the same time they become larger in size, though diffuse in substance and rather pale in color. With the completion of synapsis, the nucleus is filled with rather broad threads or chromosomes quite uniform in staining reactions (fig. 6) and often showing a definite polar arrangement. This phase seems to be rather prolonged, nuclei showing it being very abundant.

At this time a large nucleolus, the 'Intranuclear-Körper' of Lenhossék ('98), makes its appearance in the nucleus (fig. 7). It sometimes seems to be enclosed by a distinct vesicle, the borders of which are clear cut and definite in outline, though not bounded by a distinct membrane. It occupies a peripheral position. At the time of synizesis the vesicle seems to fill most of the space left by the clumping of the chromatin threads (fig. 9). The origin, as well as the fate, of this nucleolus is problematical. It is usually larger than the nucleoli in the preceding stages and is composed of two parts, one of which stains much less intensely than the other (figs. 9 to 12). Lenhossék figures it as a lightly staining matrix with a few small darkly staining granules, most of which are arranged around the margins. It increases in size rapidly and, in the late pachytene stage, may attain a length of two-thirds the diameter of the nucleus. The vesicle disappears in the diakinesis and the nucleolus becomes smaller in size, but it is impossible at the present time to say whether it becomes one of the chromosomes or is resorbed, as its further history has not been traced.

Synizesis begins as a drawing together of the chromatin threads, while at the same time they thicken and take a darker stain (figs. 7 to 9). The clumping is not extreme, but the threads become less distinct in outline than in the preceding stage. One or more nucleoli can usually be found embedded within the mass of chromatin threads (fig. 9).

An increase in size both of the nucleus and the cell begins at the end of synizesis. The beginning of the unraveling process is a somewhat confused phase in which the chromosomes seem to be indistinct and broken up (figs. 10, 11), but these soon reappear as typical pachytene threads (figs. 12, 13). These take a darker stain and are rather broad, granular bands with a fairly regular outline.

The diplotene phase shows the characteristic opening up of the chromosomes with the formation of loops and twisted threads with processes streaming out from their borders (figs. 14 to 16). These soon condense into the typical tetrads (figs. 19, 20). During this change they begin to take a heavier stain and their borders become smoother in outline, though fine, short threads still remain attached to them (figs. 17, 18). These threads have all disappeared before the spindle is formed.

With the formation of the tetrads, the number of chromosomes may easily be counted, some cells containing twenty-one (fig. 19), and others thirty-one (fig. 20) in the mixed strain of rats. This change evidently occurs at the very beginning of the maturation processes when the leptotene threads are being formed, as there is no break in the usual procedure from this time on.

No attempt has been made to trace the sex chromosomes through these early maturation phases. In the division of the secondary spermatocytes they appear as an *xy* pair, one part of which is much larger than the other (Swezy, '28). A more careful study of the two or more nucleoli, including the large one, which persist throughout the early maturation phases must be made before anything definite can be said about the early history of this chromosome. If, as is quite probable, its history is the same as in man (Evans and Swezy, '29), we would expect to find it differentiated in the early phases as one of these nucleoli.

In material fixed with Bouin's fluid the 'Sphäre' of Lenhossék or 'idiozome' of Duesberg, a rounded body lying in the cytoplasm, does not stain with iron hematoxylin. It appears as a homogeneous body of a pale color, quite distinct from the surrounding cytoplasm (figs. 12 to 15), though not darker in color. In material fixed with Gilson's solution it presents a striking appearance (fig. 17). The body is only very lightly stained, but around its borders are arranged short rod-like masses of darkly staining material—the Golgi bodies. These rod-like masses vary somewhat in size, most of them being rather heavy. They do not usually completely surround the body and seem to be slightly embedded in it. In the center are two rather large granules placed side by side.

In the early stages of the primary spermatocytes this body appears as an elongate clump of rods lying in the cytoplasm near the nuclear membrane. This gradually enlarges until at the beginning of the confused stage it is a rounded body (fig. 10) of considerable size. It reaches its greatest size during the diakinesis. At just what stage the two granules or centrosomes leave their position in the center of this body it is difficult to say, but it is not until the late diakinesis. With the formation of the spindle, the centrosomes are found at the poles, while near the outer margin of the cell the remains of the body may occasionally be found.

A comparison of the maturation phases in the male germ cells with those of the female germ cells in these rats reveals some striking differences. In the embryonic female germ cells the classical model of maturation is followed throughout up to the beginning of diakinesis, which does not ordinarily occur in these cells (Swezy, '29). The synaptic phase between the formation of the leptotene threads and synizesis is greatly prolonged in the male germ cells (figs. 4 to 8), the same phase being much less in evidence in the female. The picture presented by synizesis in the male is not quite the characteristic one that is found in the female, and the confused stage which follows it (figs. 10, 11) is also absent in the ova. The pachytene is the same in both lines of cells.

It is in the germ cells of the adult female rat, however, that the greatest variation exists. As has been pointed out elsewhere (Swezy, '29), synapsis, synizesis, and the pachynema have been dropped out as distinct phases in the maturation of the ova, and a substitute phase developed which evidently combines the results of the phases thus eliminated. In the male germ cells the stage of synapsis and synizesis differ somewhat from the typical pattern, yet these phases are rather prolonged. Another modification is introduced in the presence of a confused stage between synizesis and the pachynema. In some forms (Wilson, '25) a confused stage occurs after the pachynema, but the position of the cells leaves no doubt here that it occurs before the pachytene threads are formed. Both the pachynema and diakinesis present the typical characteristics throughout the further development of the chromosomes. The spermatocytes and oocytes in the mixed strain alike produce individual cells, some of which have twenty-one and some thirty-one tetrads, with no evidence in either case of the cause or the point at which this change occurs.

Summing up these differences and agreements, we find that at only two points are there any resemblances between the maturation of the male and the female germ cells of the rat. The first point is the deutobroch nucleus, which is much the same in both lines of cells. The second is found when the spindles are formed and the tetrads take their places upon it, with two distinct chromosome numbers in each sex in the mixed strain. The two lines of cells have thus followed widely differing paths, but the beginning and the end are the same in both cases.

SUMMARY

The male germ cells of the rat show only slight modifications in the early phases from the typical procedure in their maturation phenomena. A rather prolonged period of synapsis precedes synizesis. The clumping in the latter phase is not extreme. A confused stage follows synizesis from which the typical pachytene threads emerge.

A greatly enlarged nucleolus is conspicuous during the middle phases, but both its origin and fate are unknown.

The centrosomes are embedded in an idiozome which does not stain after Bouin's fluid, but does after Gilson's. With this fixative it is seen to be surrounded by Golgi bodies.

Maturation of the male germ cells resembles that in the female germ cells in the same strain of rats only at the beginning and end of the process, i.e., the deutobroch nucleus and the tetrads as they appear on the spindle.

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EXPLANATION OF PLATES

All figures have been drawn from rat testes fixed with Bouin's fluid and stained with iron hematoxylin, except figure 17, which was taken from material fixed with Gilson's fluid. Uniform magnification of 2650 diameters.

PLATE 1

EXPLANATION OF FIGURES

1 Spermatogonium showing nucleus in the resting phase.

2 Primary spermatocyte with an early deutobroch nucleus.

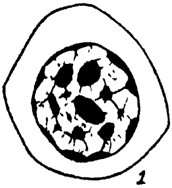
3 Leptonema.

4 to 7 Stages in synapsis.

8, 9 Synizesis.

10 Beginning of the confused stage. The idiozome in this and the following figures is represented by a circle.

11 Confused stage.



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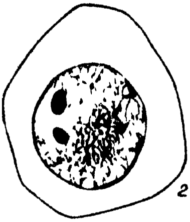

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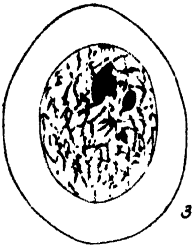

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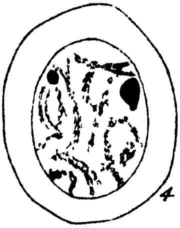

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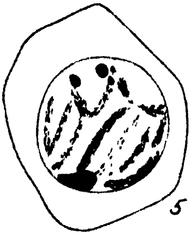

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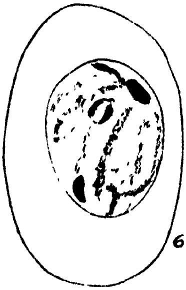

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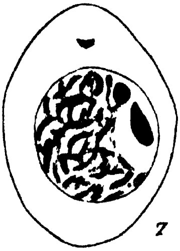

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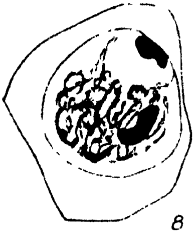

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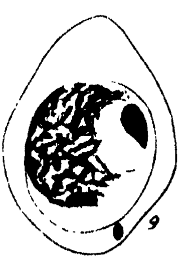

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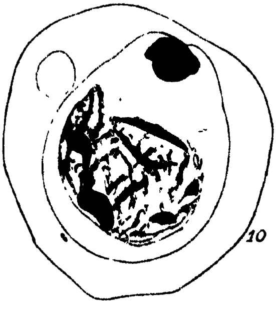

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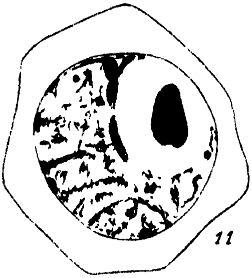

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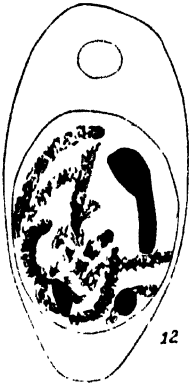
PLATE 2

EXPLANATION OF FIGURES

12, 13 Pachynema. Note greatly enlarged nucleolus.
14 to 16 Diplonema.
17 Early diakinesis. The idiozome shows the surrounding Golgi bodies.
18 Later diakinesis with the tetrads becoming more condensed.
19 Late diakinesis with thirty-one tetrads present.
20 Late diakinesis with twenty-one chromosomes present.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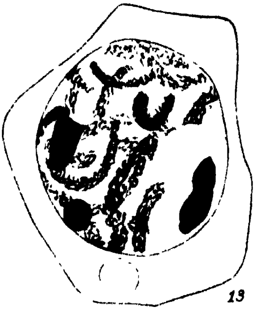
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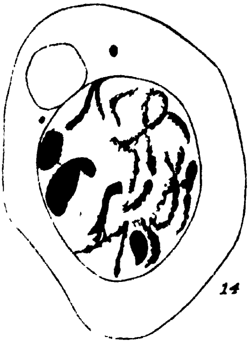
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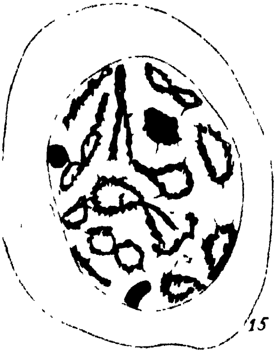
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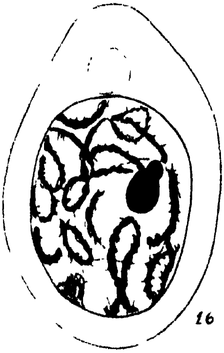
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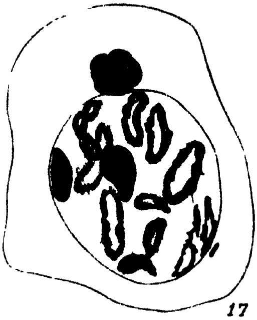
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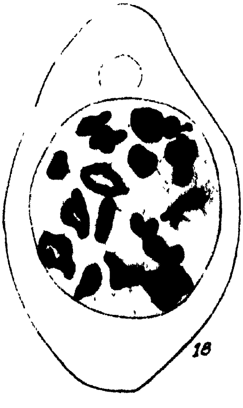
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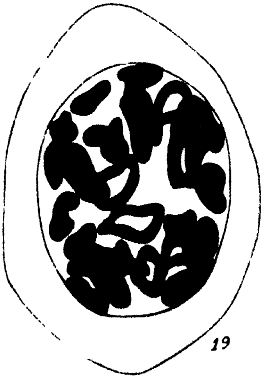
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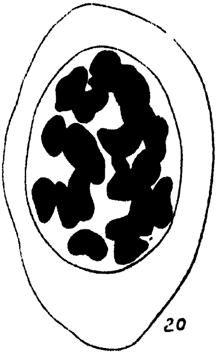
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THE OVARIAN CHROMOSOME CYCLE IN A MIXED RAT STRAIN[1]

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THREE TEXT FIGURES AND TWO PLATES (TWENTY-FOUR FIGURES)

AUTHOR'S ABSTRACT

In the ovary of the rat the ova arise by proliferations from the germinal epithelium, all cells of which are potential ova This proliferation begins with the differentiation of the gonad, and may last as long as 369 days postpartum The embryonic ovary is filled with ova which pass through the typical maturation phases This continues until five days after parturition These ova degenerate, none being found in the ovary of the twenty day rat. After the fifth day postpartum, nuclear development in the ova changes until, by the twentieth day, no typical maturation phases are present With the degeneration of the embryonic ova the ovary takes on the adult structure

The ovary of the adult female rat shows a modified type of meiosis in the germ cells, while that of the embryo shows the typical phases, indicating that this is the primitive type, with the modified form an acquired characteristic

Ova in a single rat may show both twenty-one and thirty-one chromosomes.

Follicle cells are formed from the cells of the germinal epithelium and, like the sex cells, may have both forty-two and sixty two chromosomes in a single follicle. The lutein cells also show both forty-two and sixty two chromosomes in a single corpus luteum The chromosomes of the lutein cells enlarge with the expansion of the cell to a size greatly in excess of the chromosomes of the somatic cells The theca interna is derived from the tunica albuginea

CONTENTS

Introduction 445
Material and methods 446
Embryonic ova 447
Origin of definitive ova 450
Chromosomes 454
    Sex cells 454
    Follicle cells 459
    Lutein cells 460
Discussion 463
Summary 467
Literature cited 469

INTRODUCTION

In an earlier paper (Swezy, '28) the presence of two distinct chromosome counts in a mixed rat strain was noted,

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[1] Aided by grants from the Committee for Research in Problems of Sex of the National Research Council, administered by Prof. Herbert M. Evans, to whom the author is greatly indebted.

the material studied consisting of somatic male and female embryonic cells and male sex cells. As a continuation of these studies, the female reproductive cells of the same group of rats were examined, the results of which are given in the following pages.

Much uncertainty still exists as to the methods of oogenesis in the vertebrates, particularly in the mammals. The most careful recent work has shown that this process differs in many important details from that found in the invertebrates. It has also been shown that oogenesis differs from spermatogenesis in mammals, even in the same species—a result which seems to contradict the text-books of cytology where these two processes are figured as identical until the final maturation divisions occur. Kingery ('17) finds that the early maturation phases do not occur in the ova of the mouse —which is a radical change from the generally accepted theory of oogenesis. The origin of the great number of germ cells which are present in the embryo at about the time of birth in the rodents at least is no longer a mystery. It has been shown that these arise by proliferations from the germinal epithelium, and not by repeated divisions of a few original germ cells.

The recent work of Simkins ('28) on the origin of the sex cells in man traces the development of these from the germinal epithelium from the earliest fundaments onward until the formation of the tunica albuginea prevents further ingress of cells from this source. He, however, leaves untouched the problems of oogenesis.

The rat, because of the ease with which it may be handled, its great fecundity, and the exact data accumulated on its periods of gestation, etc., is an especially favorable subject for the investigation of these problems.

MATERIAL AND METHODS

The animals used for these studies were members of our rat colony, the origin of which, as well as the special technique used, was given in an earlier paper (Swezy, '28). These included embryos of varying ages up to birth and thereafter to twenty days postpartum, and another group beginning at twenty-nine up to 369 days of age. The latter may be further divided into three groups, the first of which was the young, unmated rats; the second, the pregnant rats, and the third, rats killed at definite intervals during the normal oestrous cycle.

For the study of the lutein cells the pregnant females were used, and these ranged in time from one day up to full term. For comparison with these the ovaries of a number of guinea-pigs were used, fixed and stained by the same methods that were followed with the rats.

EMBRYONIC OVA

The development of the ovary from the undifferentiated gonad is well advanced at the middle period of embryonic life. From this time on, the primitive ova may be easily differentiated from the surrounding cells by their slightly larger size and rounded nuclei.

The formation of embryonic ova from the germinal epithelium is a continuous process up to the time of birth, and is identical in all aspects with that figured (fig. A, 1 to 3) for the development of the definitive ova in rats nearing sexual maturity. The differentiation of the ovum is not marked by a special mitotic division as Allen ('23) describes in the mouse, any cell in the germinal epithelium apparently being a potential ovum. Mitotic divisions are usually quite abundant, but the products give no indications of any change from the epithelial character of the parent cell. The early differentiation of the ovum consists of a change in the form of the nucleus, which becomes vesicular, while the cytoplasm also tends to assume the same form, along with a gradual enlargement of both. The chromatin network becomes more distinct, with chromatin granules lodged at the intersections and among them one or more nucleoli. Cells in this phase with a deutobroch nucleus may be seen migrating from their position in the germinal epithelium to the regions below.

About the sixteenth day, a series of nuclear changes becomes evident, similar in appearance to those figured by Winiwarter and Sainmont ('08) in the embryonic ova of the cat and by Kingery ('17) in the embryonic ovary of the white

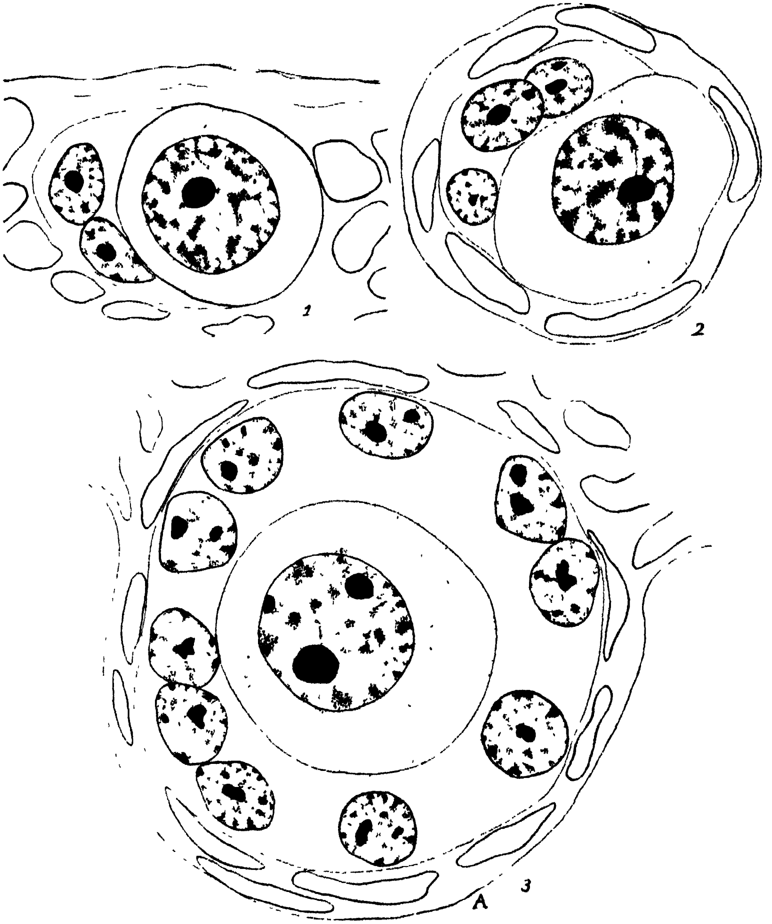

Fig. A 1. Newly formed ovum in the germinal epithelium. Two follicle cells attached to it and separating from the other epithelial cells. Surface of epithelium is at the top. 2. Ovum in the tunica albuginea with three follicle cells and the theca interna present. 3. Primary follicle migrating from the tunica albuginea with the theca interna fully formed. Uniform magnification of 2000 diameters.

mouse. These changes are identical with the early maturation phases of the spermatocytes of other forms, hence were included in oogenesis by Winiwarter and Sainmont. They are also identical with those of the five-day rat shown in figures 1 to 5.

The leptonema is perhaps most striking at about the eighteenth day, in some cases all or nearly all the ova showing this phase of nuclear change. The number of leptotene threads is large and is evidently the diploid number. This is followed by synizesis with the threads massed in a dark clump near one side of the nucleus.

By the twenty-first day, the majority of ova present in the embryo show the pachynema and diplonema stages, though earlier stages, including newly forming ova in the germinal epithelium, may also be found.

There is but little evidence of follicle formation at this time, the ova lying in clumps with a rather scanty amount of connective-tissue cells scattered among them. An occasional primary follicle with a single layer of cells may be seen, but these do not have the distinctive appearance they present in the ovary nearing sexual maturity.

Ova may occasionally be found in which attempts at division are being made, with the chromatin formed in numerous, small, round granules or chromosomes. These are irregular in number. The same phases have been described in the human testis (Evans and Swezy, '29) and are evidently a form of degeneration, as no instances of successful division in these cells have been found.

At the time of birth the ovary, though small, contains more ova than is present at any later period of life, Arai ('20) having found about 35,105 in the rat one day old. The organ is so completely filled with the ovarian cells that there is no space for developing follicles and the characteristic medulla and cortex, none of which becomes well developed until degeneration of the embryonic cells is well under way.

Embryos earlier than sixteen days were not examined. According to Pratt and Long ('17), oogonial divisions are numerous up to this time, the last divisions occurring at about fifteen days, after which the maturation phases begin. Their account of these earlier nuclear changes is more complete than has been given above, and the reader is referred to their paper for these details. The serial appearance of the different stages up to the time of birth in our material also agrees with their findings.

About the origin of the embryonic ova which are already present at the sixteenth day nothing can be said. From the sixteenth day on, however, it is certain that ova are being constantly differentiated from the germinal epithelium, and, as maturation immediately sets in before they have left the germinal epithelium, there are no further opportunities for oogonial divisions to take place. The greatest increase in the size of the ovary occurs between the sixteenth and twentieth days, and there is a corresponding increase in the number of ova which it contains. Since the oogonia no longer divide, this increase in the number of ova must come only by way of new proliferations from the germinal epithelium.

In his work on the human ovary, Simkins ('28) found that all the fundaments, including the germ cells, of the ovary were derived from the germinal epithelium, and it is probable that the origin is the same in the rat, since the later history up to the time of birth is the same in both cases.

ORIGIN OF DEFINITIVE OVA

The origin of the definitive egg cells or ova from the germinal epithelium has been described by a number of investigators, among whom may be mentioned Lane-Claypon ('05) in the rabbit, Winiwarter and Sainmont ('08) and Kingsbury ('13) in the cat, Kingery ('17) in the white mouse, Robinson ('18) in the ferret, Arai ('20) and Butcher ('27) in the white rat, Allen ('23) in the mouse, and Papanicolaou ('24) in the guinea-pig.

The time during which oogenesis takes place has been a much-discussed question, the generally accepted view being that this does not occur after sexual maturity. A complete review of the literature on this subject may be found in Allen's ('23) excellent paper on the mouse. A few names only will be mentioned here. Winiwarter and Sainmont ('08) describe three separate proliferations of germ cells from the germinal epithelium, the first two of which degenerate, the third occurring at the age of about three and a half or four months and forming the definitive ova, the process being limited to a brief period of time before sexual maturity.

Kingery ('17) finds two proliferations of germ cells in the white mouse, the first occurring before birth, the second extending from birth or a few days later nearly to sexual maturity. The first proliferation gives rise to the embryonic germ cells which degenerate and are absorbed, the second forming the definitive ova.

Robinson ('18) found the process in the ovaries of the ferret essentially the same as that described by Kingery in the mouse, but also states that his evidence shows that new groups of ova and follicle cells are formed throughout the whole period of the functional life of the ovary.

The observations of Arai ('20) on the white rat place the beginning of the formation of the definitive ova from the germinal epithelium at ten to fifteen days after birth, continuing actively to sixty days, when the process slows up, though it may continue for a year after birth.

Allen ('23) finds that in the mouse "a cyclical proliferation of the germinal epithelium gives rise to a new addition of young ova to the cortex of the adult ovary at each normal oestrous period," and that this continues to take place during the entire period of sexual maturity. This view has been confirmed in essential points by Papanicolaou ('24) in his work on the guinea-pig. He concludes that oogenesis is a continuous but rhythmical or cyclical process from the time of gonadal differentiation up to the time of cessation of sexual activity.

The work of Arai ('20) has shown that the total number of ova is greatest during the first days after birth, some containing as many as 35,105, the number decreasing rapidly until it drops to about 11,000 at twenty days. This change can be accounted for only by a rapid process of degeneration in which most, if not all, of the embryonic ova disappear. The new generation of oocytes begins to form in the germinal epithelium before five days after birth.

The formation of true follicles was first observed in the five-day ovary. The central part of the ovary is at this time fast losing its embryonic character, and a large part of the embryonic ova in this region have disappeared. By the eighth day, these changes have become more noticeable. Only a few of the ova still remain in the central region, which is fast developing the typical medulla and cortex of the mature ovary. Many atretic follicles are present. Whether these follicles are some that have arisen since parturition or are the remains of the embryonic follicles cannot be said.

The ovary by the tenth day has lost nearly all its embryonic characteristics. The ova have entirely disappeared, except in a zone under the germinal epithelium. This zone is rather wide and is closely packed with ova, most of which show the beginning of follicular development.

By the fifteenth day, the medulla and cortex are well developed and the young ova are restricted to a still narrower zone along the germinal epithelium. Many medium-sized follicles are present, some showing the beginning of the follicular cavity. By the twentieth day, the change into the mature structure is completed, though no corpora lutea are present. The follicles are large, with well-developed cavities, and the young ova are scattered through a very narrow zone. From this time on, no further change is noticed other than the cyclical ones common to the functional organ.

The development of ova from the germinal epithelium has already been described in the embryonic ovary, and this process differs in no way from that which takes place in later life. In rats five days after birth newly forming ova are to be found in considerable numbers. Rats from this age on to about ninety days showed ovarian cells in the germinal epithelium in great abundance. Older rats up to 369 days gave the same picture, but the number produced thus was progressively fewer in the rats of more advanced age. Rats older than these were not examined.

The number of newly forming ova present in the ovary of the rat at five days postpartum, both in the germinal epithelium and immediately below, indicates that this process began at a still earlier age. At the time of birth it has not ceased, and probably continues without a definite break, though in a more or less cyclical manner. Nothing, however, can be said definitely about the period between this and five days, as no examinations were made here, yet it seems highly probable that it does not altogether cease.

While the ovum is still in the germinal epithelium one or more cells closely associated with it are found to be slightly differentiated from the surrounding cells. The cytoplasm becomes clearer and more definitely bounded, the nuclei slightly more vesicular, and the cells become closely adherent to the ovum (fig. A, 1). These are the first follicle cells and are closely related to the ovum itself through direct cell division of the earlier germinal epithelial cells. Apparently, but one or two cells, usually one, are derived directly from the epithelium, the primary follicle being the result of divisions of these which spread (fig. A, 2) out until they enclose the ovum. These first divisions are passed through rapidly with no resulting constriction of the cytoplasm, the primary follicle usually appearing as a syncytium (fig. A, 3) closely adherent to the wall of the ovum. These divisions take place after the ovum has migrated from the germinal epithelium into the tunica albuginea, and the primary follicle may be fully developed before it leaves the tunica (fig. A, 3).

As the ovum passes into the tunica albuginea some of the cells of this layer attach themselves to it, forming the theca interna (fig. A, 2). These cells become distinct from the other tunica cells and are found with their nuclei close to the wall of the growing follicle and stretching around it until it is completely invested. This may be completed before the follicle leaves the tunica albuginea (fig. A, 3). In many cases the primary follicle is not fully formed until the ovum has passed through the tunica, and the theca may likewise consist of only a partial layer of cells. These divide to keep pace with the growing follicle.

The newly forming ova and primary follicles do not wander far from the germinal epithelium, but usually occupy a narrow zone that extends around the entire ovary. In the region around the hilum they are more numerous than in other parts. As a follicle enlarges it makes room for itself by pushing outward in lobules, the germinal epithelium growing rapidly to keep pace with this new demand upon it. Very few ova are to be found developing from the germinal epithelium bounding follicles or corpora, probably due to the tension put upon these parts by the expanding body beneath.

CHROMOSOMES

The presence of two distinct chromosome numbers in the spermatocytes of this group of mixed rats and the finding that these would, apparently, mate only with ova having a like number (Swezy, '28) led to the conclusion that two distinct chromosome numbers would also be found in the ovarian cells in the same group of rats. Examination of ovarian material abundantly confirmed this, but also disclosed a curious and seemingly inexplicable situation in chromosome behavior in other parts of the ovary. These will be taken up in detail in the following paragraphs.

Sex cells

The development of the sex cells in the embryo has been described in earlier paragraphs. These follow the classical model up through the leptonema to the dictyate stage, which has many of the characteristics of a resting phase. This is followed by degeneration beginning at the time of birth. In the sex cells which arise at later periods these early maturation phases are obscure, if not entirely omitted, after maturity is reached.

The formation of new ova from the germinal epithelium is apparently a continuous process, as it occurs abundantly in rats of 5, 8, 10, 15, 18, and 20 days postpartum. Rats between these days were not examined. The process is identical with that already described for the embryonic ova and is shown in some detail in figure A, 1 to 3. The development of the follicle is dealt with in later paragraphs.

The ovum enlarges to several times the size of the original germinal epithelial cell (fig. A, 1, 2), and then may remain stationary in size for a considerable period of time, as the large number of such ova immediately below the germinal epithelium would seem to indicate. The final growth period is fairly rapid.

Nuclear phenomena in these ova which arise after birth present a progressively differing series of changes in the period between five days after birth and the time sexual maturity is reached. In the ovary of the rat of five days the newly forming ova pass through all the maturation phases (figs. 1 to 5), beginning with the leptonema while still in the germinal epithelium. By the time the ovum has passed through the tunica albuginea, synizesis is in progress (fig. 3). This is followed by the pachynema, in which the chromosomes present the typical appearance for such stages—rather broad, granular threads, gray in color, except where seen vertically, when they appear black (fig. 4). A nucleolus is present usually near the side from which most of the threads radiate. In ova only slightly larger than these the diplonema may be found, in which the nucleus becomes filled with a loose arrangement of chromatin threads, many of which are distinctly paired (fig. 5).

In the ovaries from rats of eight days the nuclear picture presents a decided change (figs. 6 to 9). The germinal epithelium contains numerous ova, many of which show a leptotene arrangement of the chromatin. Synizesis stages are much less abundant than in the five-day rats, and in those that do occur the picture they present is less typical. The chromatin threads are not so distinct and the ball or clump is much larger in size and usually not so closely related to the nuclear wall (fig. 7).

The stage following this is evidently the pachytene, but is very considerably modified in appearance (fig. 8). The nucleus becomes filled with loose, granular clumps, some of which bear a slight resemblance to the ordinary pachytene chromosomes. These may or may not be connected by granular threads. The ova, which, from their size and position, would correspond to the diplonema phase in the five-day rats, show an appearance slightly modified, but undoubtedly diplotene in character (fig. 8). The chromatin threads are often lost in granular clumps for part of their lengths, but the ends show a paired arrangement. In other ova of the same category these threads seemed to have entirely broken up into granular masses.

The ovaries of the rat of ten days are quite similar in the nuclear picture they present to that of the rat of eight days (figs. 10 to 13). When the rat of fifteen days is reached, only a few further changes are to be noticed (figs. 14 to 16). The clumping in the synizesis stages is much less noticeable, only a slight loss of size being found in the chromatin contents of the nucleus, this being sufficiently evident, however, to identify it.

In the ovary at twenty days nearly all traces of the characteristic maturation phases have disappeared (figs. 17 to 22). The newly forming ovum in the germinal epithelium shows the nucleus only slightly changed from that of the surrounding epithelial cells. It is larger and spherical, but still rather pale in color (fig. 17). The chromatin network becomes heavier and darker and is lost in the loose dark granular clumps that are formed. These clumps are quite large (fig. 19), and in general appearance the nucleus resembles that appearing after synizesis in the eight-day ovary (fig. 8). Further changes in the nuclei show the gradual disappearance of the clumps, the nuclear space being filled with a very irregular network of threads along which are often many very small dark granules (fig. 20). This is evidently the diplotene, though considerably modified from that of the five-day rat.

The nucleus at this time is undergoing a great increase in size, and due partly to this and partly to the fact that much of the chromatin seems to be losing its reaction to the stain, it becomes very much paler in color. The nucleus becomes large and more or less rounded and is usually very pale gray in color with a few blobs of chromatin in it or around the margin. The chromatin granules gradually become collected in larger, more distinct clumps, some of which may be of quite large size, leaving the network quite pale in color (figs. 21, 22).

No further changes in the nuclear picture can be detected at twenty days or thereafter. The last phase described for the twentieth day seems to be the final one which lasts until the first maturation division. The early prophase stages of this have not been observed other than that shown in figure 24, where the chromatin masses are beginning to take on the form of tetrads.

There is no evidence that these maturation phases which are thus lost in the adult germ cells have been pushed back into the germinal epithelium and take place there before emergence of the differentiated ovum. Divisions in the germinal epithelium are abundant, and the cells divide in the usual manner with the customary resting phases appearing in all non-dividing nuclei.

At the time of the appearance of the first maturation spindle two chromosome numbers are found to be present in the germ cells, some ova possessing twenty-one (fig. B, 3, 4) and others having thirty-one tetrads (fig. B, 1, 2). As is the case with the spermatocytes (Swezy, '28), no evidence is forthcoming as to the manner of this change in chromosome number in these cells. The change does not take place at the time of division, as the products of division have a like chromosome number, as shown in the metaphase where thirty-one chromosomes are separating into halves (fig. B, 2), and in figure B, 4, with the second maturation spindle and the polar body having the same chromosome number.

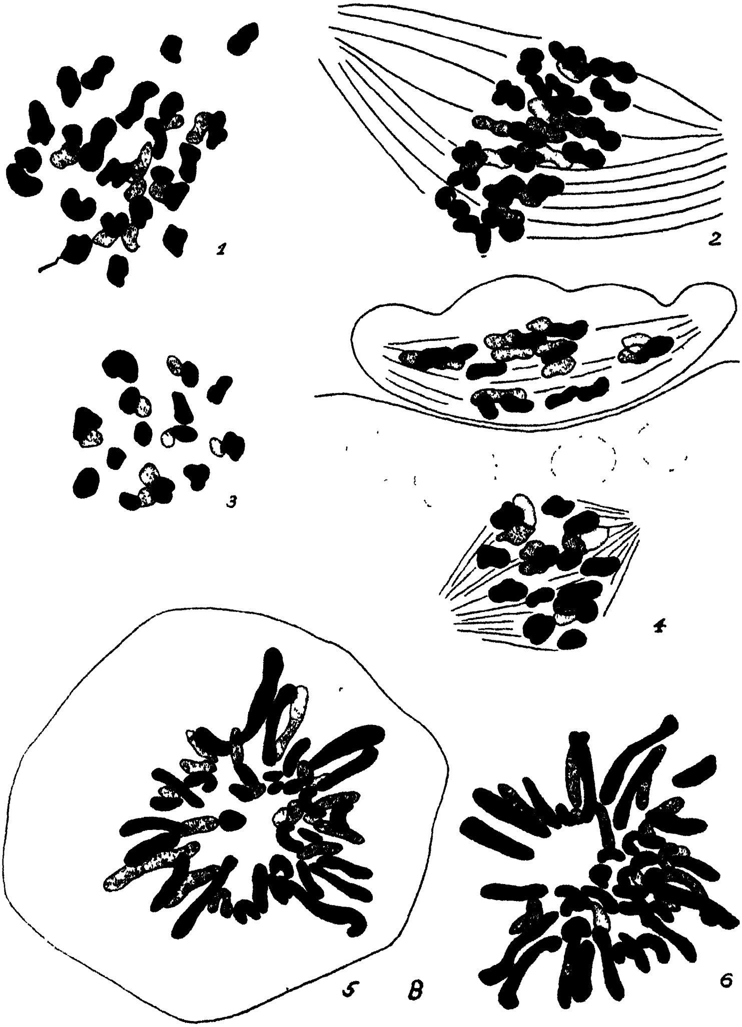

Fig. B 1. Equatorial plate of first maturation division with thirty-one tetrads. 2. Metaphase of first maturation division with thirty-one tetrads.' 3. Equatorial plate of first maturation division with twenty-one tetrads. 4. Second maturation division with spindle forming in the polar body. Twenty-one chromosomes in each. 5. Equatorial plate of a lutein cell with fifty chromosomes. 6. Equatorial plate of a lutein cell with forty-eight chromosomes. Uniform magnification of 2600 diameters.

Centrosomes could not be located in the maturation divisions of the ova, the spindle fibers having the characteristic truncated appearance shown in figure B, 2 and 4, with their ends often quite widely separated. Since, however, centrosomes are to be found in all other mitotic figures in rat tissues, it seems probable that their staining reaction here is such as to render them difficult to demonstrate with iron hematoxylin.

Follicle cells

The first appearance of follicle cells occurs while the ovum is still in the germinal epithelium (fig. A, 1). As the ovum enlarges it becomes noticeably separated from the surrounding epithelial cells, which are crowded to either side. Usually included in this space that is thus marked out are one or more of the cells from the germinal epithelium, and these become closely associated with the ovum. As the ovum passes down into the tunica albuginea these cells go with it and form the beginning of the follicle (fig. A, 1). They divide in a short space of time and spread out until they invest the entire ovum. Where these divisions have taken place rapidly no cell boundaries can be detected between the nuclei (fig. A, 3). The nuclei at this time are rounded and often have a greater resemblance to the nucleus of the ovum than to the nuclei of the surrounding somatic cells. The cytoplasm is clear and finely granular, like that of the ovum.

The theca cells are formed from the tunica albuginea (fig. A, 2, 3), the cells of which are usually elongate and rather narrow. These stretch themselves around the ovum and its accompanying follicle cells as they leave the germinal epithelium and form a close-investing covering. In some parts of the ovary the tunica albuginea appears as a wide zone and the ovum may remain here until the primary follicle is completely formed (fig. A, 3), after which it continues its journey to the zone below. In other parts of the ovary where the tunica is narrow the theca interna may not be completed before the ovum has finished its migration.

Division of the follicle cells offers no variations from the procedure in other cells so far as methods are concerned and will not be considered in detail. Counts were made on the number of chromosomes in follicles at all stages of their development, both in prophase and equatorial-plate stages. It was found that a single follicle produced cells some of which had sixty-two chromosomes, while others had forty-two. No attempt was made to determine the relative number of these counts in a single follicle. Figure C, 3 to 5, was drawn from cells of the stroma, but may equally well be used to illustrate the two types of chromosome counts in follicle cells, though the prophase is usually more compact in the latter, as the cells are more closely packed together.

Dividing cells are generally abundant throughout all stages of growth of the follicle, ceasing at about the time of the change of these cells into lutein cells.

True follicle cells have not been found in the embryonic ovary. Many of the ova show a few cells encircling them, but, as has been pointed out earlier, these do not have the appearance of primary follicles, as these are found in the mature ovary. The probable reason for this is shown in the development of the follicle as it has been given above. In the embryonic ovary the tunica albuginea does not develop until a relatively late period when the ovary is already filled with ova. The so-called primary follicles are found only near the central regions, far removed from the germinal epithelium. It seems quite likely that these cells are connective tissue and 'indifferent' cells which have pushed in among the ova until some have become completely surrounded.

Lutein cells

It is in the lutein cells that the most striking appearance in these chromosome variations occurs. Dividing cells were found in the corpora lutea of pregnancy between the tenth and fifteenth days. They were searched for at other periods, beginning with the first day of pregnancy up to full term, but

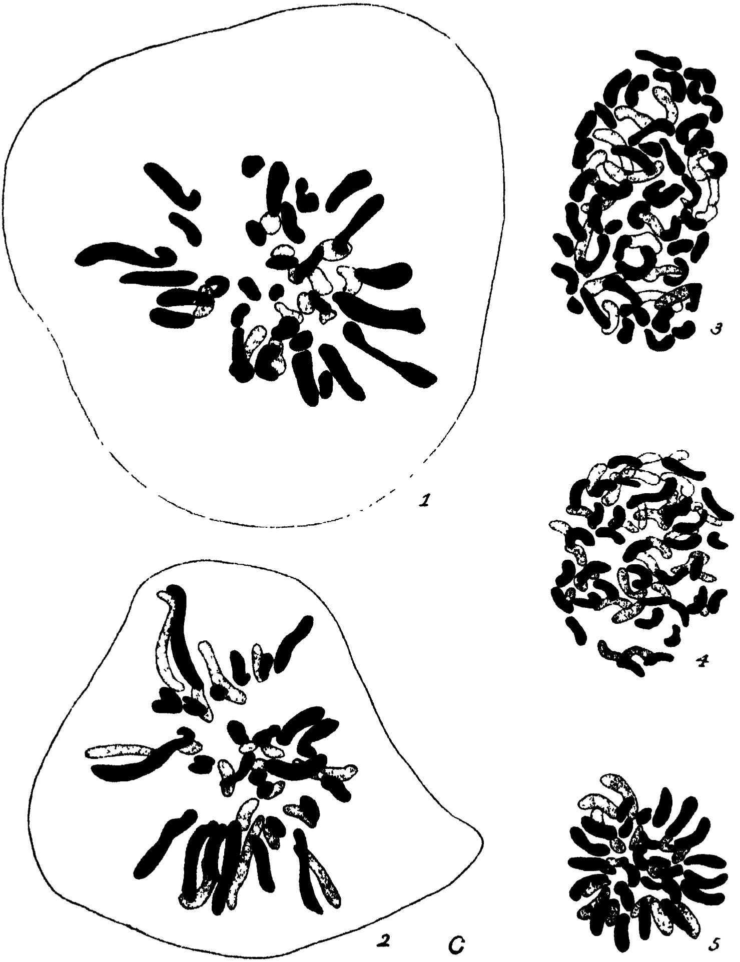

Fig. C 1. Equatorial plate of a lutein cell with forty two chromosomes. 2. Equatorial plate of a lutein cell with forty five chromosomes. 3. Prophase of a cell from the stroma with sixty-two chromosomes. 4. Prophase from the stroma with sixty-two chromosomes. 5. Equatorial plate of a cell from the stroma with forty-two chromosomes. Uniform magnification of 2600 diameters.

with no success. In most cases they were not abundant, but occasionally three or four would be found in one microscopic field. These may be searched for with the lower powers of the microscope, as the dividing cells are very conspicuous.

The corpus luteum attains its maximum size usually at about the sixteenth day. The cells at this time are large and clear, with little or no darkly staining material such as appears in the cytoplasm around the eighteenth day and later. The cell size greatly exceeds the size of any other cells in the ovary, aside from the ova themselves. This increase in size may be noted as a gradual process starting with the follicle cells.

At the time of division the cells undergo a slight further enlargement, which, together with the clearness of the cytoplasm, renders them quite conspicuous among the neighboring cells.

The chromosomes of these cells have also increased in size proportionate with the increase in cell size (fig. C, 1, 2). A comparison of these with chromosomes from other tissues (fig. C, 3 to 5), all of which were drawn at the same magnification, shows that the increase has been about double in length and rather less in thickness, and extends throughout the entire series of chromosomes.

These enlarged chromosomes are found in all the various stages of mitosis, which gives every indication of being a perfectly normal process, no signs of degeneration being detected in the numerous cells which have been examined. Neither are degenerating cells or evidences of them present in the corpora lutea of this period. The number of long and short chromosomes remains the same as in the other tissues in cells where either forty-two or sixty-two are present. As with the follicle cells, both these chromosome numbers may be present in the same corpus. These numbers are not constant, however, variations ranging from thirty-five to sixty-two having been found (fig. B, 5, 6)—differing in this respect from the follicle cells, where no variations were encountered. These variations in number other than the two basic ones are evidently due to fragmentation and to the fortuitous union of two or more chromosomes such as are sometimes found in other forms.

DISCUSSION

Kingery ('17) questions the appropriateness of the term oogenesis for these phases of development in embryonic ova, since they degenerate and do not form the definitive ova. It is well known that a far greater number of ova degenerate than ever come to maturity, and this continues throughout the period of ovarian activity. Evidence seems to show that this period of ovarian activity begins early in foetal life before conditions favorable for the complete maturing of the ova are present, and this lack leads to their degeneration soon after birth. This lacking stimulus is not supplied until the period of sexual maturity is reached, during which interval ova are constantly forming and degenerating in great numbers. The embryonic ova have the same method of origin as do the ova formed later in life and are as truly ova as are the thousands of cells which later develop into primary and mature follicles and then degenerate.

Winiwarter and Sainmont ('08) distinguish three distinct periods of proliferations of ova from the germinal epithelium in the cat, and Kingery ('17) two in the mouse. In the rat evidences seem to point to one continuous proliferation which begins with the differentiation of the gonad and ends only with the final cessation of sexual activity. This may be more or less rhythmical, but seems never to wholly cease, as no single ovary has been found without some evidences of such proliferation.

The differences noted in the maturation processes in these germ cells are not easy to explain. These processes in the embryonic ova are long drawn out and hundreds may be found in all the various stages. This condition continues until after the fifth day postpartum, when the maturation phases become gradually modified until in the adult they have become shortened almost, if not quite, to the point of extinction as distinct phases. It is evident that some influence can be operative at the time of maturation of the embryonic ova without, at the same time, influencing the developing ova in the maternal organ, for the latter never show the typical maturation phases during the period of pregnancy or at any other time.

Kingery ('17) finds the same lack of definite early maturation phases in the ovary of the mouse and suggests that synizesis may represent a stage in degeneration. He further quotes Duesberg's ('08) report that synizesis does not occur in the spermatogenesis of the rat. However, the occurrence of these phases in the final division of the germ cells, sometimes in more or less modified form, has been established by careful, painstaking work in too many forms to be thus dismissed.

In the attempt to find some explanation for this unusual phase of development in the rat, both the testis of the rat and the ovary of the guinea-pig were examined. In the former the findings did not agree with those of Duesberg cited above. In the maturation of the spermatogonia only a very slight modification of the typical pattern has been found (Swezy, '29). This modification is most noticeable in the earliest stages, the leptonema and synizesis showing the greatest variation. These, however, are recognizable stages. The pachytene and later stages are typical in every respect. There is thus much less change in the maturation pattern in the spermatogonia than in the oogonia. None of the stages in the one line of germ cells shows any close similarity to those of the other line.

An examination of the ovary of the guinea-pig shows that synizesis (of a type shown in fig. 7) takes place as soon as the ova reach the zone below the germinal epithelium, though occasionally such a stage may be found in the germinal epithelium itself. These phases are quite abundant, indicating that they are of rather long duration, while considerable search is necessary to find other stages. The diplonema, with slender chromatin threads, some of which are paired, is found in the primary follicles. Other phases are rare or missing altogether. Comparing these with conditions in the rat, it is found that the type of maturation of the ova in the adult guinea-pig is quite similar to that found in the rat of eight days.

The development of the sex cells in these rodents thus presents a curious phenomenon. The process of meiosis is modified in the adult and to a much greater extent in the female than the male. Along with this modification we find in the development of the embryo ovarian cells which pass through all the typical phases of meiosis in their most perfect form. The ova which develop in the maturing animal show the modified type of meiosis. This is evidently an acquired characteristic developed late in ontogeny, the embryo showing the primitive condition from which this modified type is derived by dropping out certain stages and changing others to a greater or less extent. Also, this change from the primitive or basic type may be observed taking place in the ovary of the rat from five to ten days postpartum. What factors condition these changes cannot even be surmised.

A comparison of the chromosome numbers in the cells of the ovary with those of the testis in the same group of rats also presents a curious contrast. In the testis the only variation in number is found in the primary and secondary spermatocytes (Swezy, '28), which show the haploid numbers of twenty-one and thirty-one. The cell generations immediately preceding these, the spermatogonia, do not show variations in the number of their chromosomes. The chromosome counts in the ova agree with this. The haploid numbers of twenty-one and thirty-one are found in the last two divisions. No divisions such as are found in the spermatogonia occur in the oogonia, as the last divisions known here are those that take place in the germinal epithelium before the cell has begun to assume the appearance of an oogonium. The behavior of the chromosomes in the germ cells is thus consistent in both sexes. In the ovary, however, other groups of cells which do not have their counterpart in the testis show the same variations in chromosome number, but in this case it is the diploid and not the haploid numbers that are present.

At first sight this seems to be a situation not easy to explain, but a consideration of the origin of the cells which show these variations offers the key to the solution of this problem. As pointed out in earlier paragraphs, the follicle cells, hence the lutein cells also, are derived from the germinal epithelium, whence the ova also take their origin. Since every cell in the germinal epithelium is a potential ovum, all the cells derived from it would be open to the hereditary influences which, in these rats, bring about the subtle chemical change in some of the germ cells which is manifested by a change in chromosome number. This is shown in the ova by the appearance of both twenty-one and thirty-one chromosomes and in the follicle and lutein cells, of forty-two and sixty-two. In other words, the investing tissue upon which the ovum is dependent and which is derived from the same source and lasts but a short time after being separated from the ovum either through rupture or degeneration, possesses some at least of the characteristics which distinguish the sex cells from the somatic and may be considered abortive ova. Since there are no cells in the testis comparable to the follicle cells in the ovary, changes in chromosome number in the male are limited to the definitive germ cells.

This situation is comparable with that found in some of the invertebrates, as, for example, in the leech, Pisciola (Jörgenssen, '10), in which the nurse cells, having a common origin with the ova, may, like them, pass through some of the maturation phases. This extreme is not reached in the follicle cells, the resemblance to the further development of the ovum being limited to a change in chromosome number.

Considering the great numbers of newly forming ova in the germinal epithelium and in the act of migrating from it to the zone below in the mature ovary, it is somewhat difficult to understand the reasons upon which earlier investigators based their claim that the embryonic ova were the definitive ova of sexual maturity. Thus Pratt and Long ('17) state that in the white rat "by three days after birth most of the nuclei have passed over into the resting stage, so-called, and in this condition they remain, with certain modifications, up to the period of sexual maturity." Kingsbury ('13) noted these cells in the germinal epithelium of the cat, but dismissed them by saying that it was unnecessary to attempt to explain them.

It seems quite probable that the total lack of maturation phases in the ovary following the first few days after birth and continuing throughout the remainder of its functional life has been largely responsible for the idea that new ova were not forming during this period. Kingery's ('17) work on the mouse demonstrated the fact that typical maturation phases of the clearness to be found in the spermatocytes of other forms are not necessary for the development of functional ova. This has been amply confirmed in the present work on the rat, where the same conditions are found. As has been pointed out above, this lack of uniformity is an acquired characteristic, and, since it occurs in the rat, mouse, and guinea-pig, it is quite probable that it will be found to occur in many other mammals.

SUMMARY

1. In the ovary of the rat the germ cells or ova arise by proliferations from the germinal epithelium. This seems to be a continuous but cyclical process which begins with the differentiation of the gonad and lasts until the rat has reached an age in some cases of 369 days.

2. The embryonic ova and those that develop in the first five days postpartum undergo a process of nuclear development identical with the early processes of maturation in the spermatocytes of other forms, including synapsis and synizesis. These cells degenerate a short time after birth. By the fifteenth day, none of the embryonic ova can be detected in the ovary with certainty.

3. Beginning soon after the fifth day postpartum, the maturation phases become modified and shortened until the adult is reached, where most of the different maturation phases are lost.

4. The adult female rat shows a modified type of meiosis in the germ cells, while the embryo shows the typical phases, suggesting that this is the primitive or basic type with the modified type an acquired characteristic developed late in ontogeny.

5. True follicle cells are apparently not formed in the embryonic ovary, the primary follicles being formed of connective tissue and indifferent cells. The true follicle cells arise from the germinal epithelium at the same time as the ovum and are sister cells with it. The theca interna is formed from the tunica albuginea.

6. Part of the ova in a single ovary possess 'twenty-one chromosomes in the maturation divisions and part possess thirty-one.

7. The follicle and lutein cells may show both forty-two and sixty-two chromosomes in the same follicle or corpus luteum. This is evidently due to the fact that, arising from the germinal epithelium with the ovum, the follicle cells are identified with the sex rather than the somatic cells and hence possess some of their characteristics. The lutein cells are derivatives of the follicle cells.

8. The chromosomes of the lutein cells reach a size greatly in excess of the chromosomes in the other cells of the ovary. They are also less constant in number, counts varying from thirty-five to sixty-two being occasionally found. This may be due to fragmentation and to the fortuitous union of two or more chromosomes.

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EXPLANATION OF PLATES

Camera-lucida drawings from rat ovaries fixed in warm Bouin's fluid and stained with iron hematoxylin.

PLATE 1

EXPLANATION OF FIGURES

Magnification, 2200 diameters

1 to 5 Nuclei of ova from ovary of rat five days postpartum.
1 Deutobroch nucleus in germinal epithelium.
2 Leptotene nucleus.
3 Synizesis.
4 Pachynema.
5 Diplonema.
6 to 9 Nuclei of ova from ovary of rat eight days postpartum.
6 Deutobroch nucleus.
7 Synizesis?
8 Stage following 7, evidently modified pachynema.
9 Diplonema.
10 to 13 Nuclei of ova from ovary of rat ten days postpartum.
10 Deutobroch nucleus.
11 Synizesis.
12 Modified pachynema.
13 Diplonema.
14 to 16 Nuclei of ova from ovary of rat fifteen days postpartum.
14 Deutobroch nucleus.
15 Modified pachynema.
16 Masses of chromatin changing into loose threads.

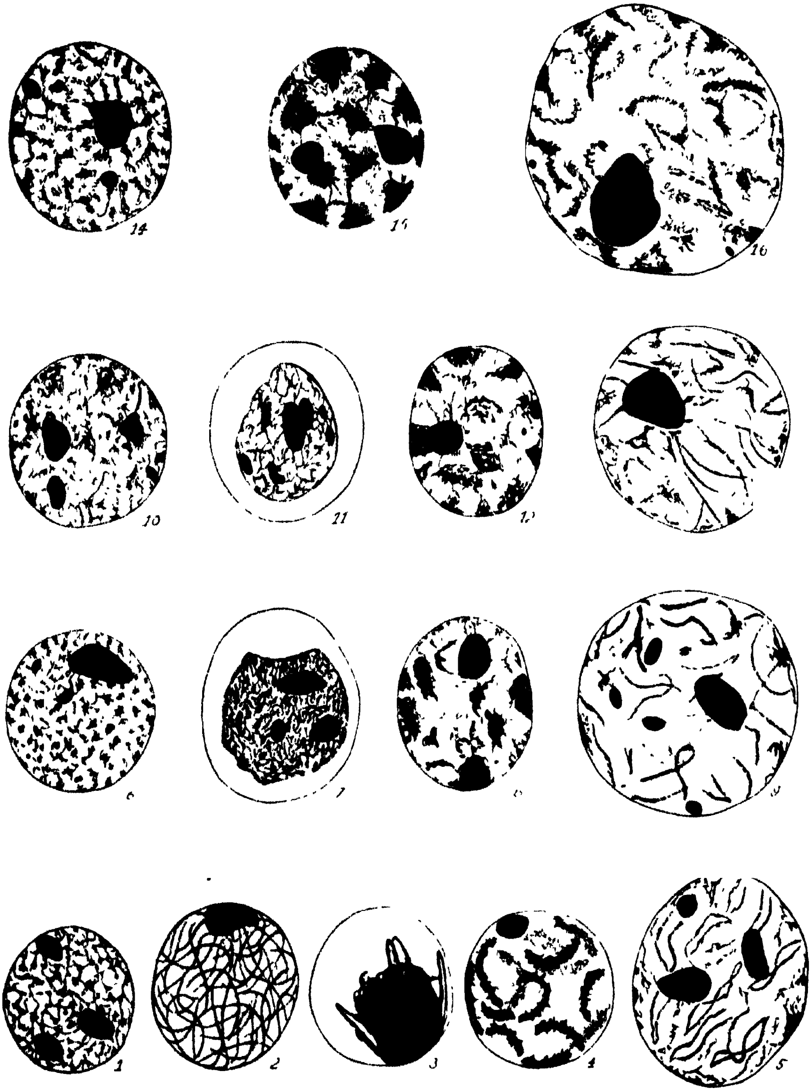
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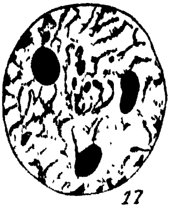
PLATE 2

EXPLANATION OF FIGURES

Magnification, 2650 diameters

17 to 22 Nuclei of ova from ovary of rat twenty days postpartum.
17 Deutobroch nucleus.
18 Beginning of the formation of clumps shown in next figure.
19 Modified pachynema.
20 Later stage showing characters of diplonema.
21 Nucleus toward the end of the growth period.
22 Final stage in twenty-day rat.
23 Nucleus from mature follicle from adult rat.
24 Nucleus from ripe follicle from adult rat.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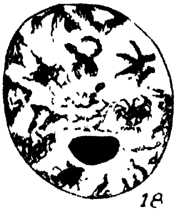
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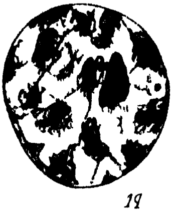
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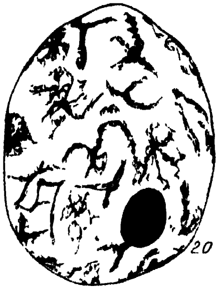
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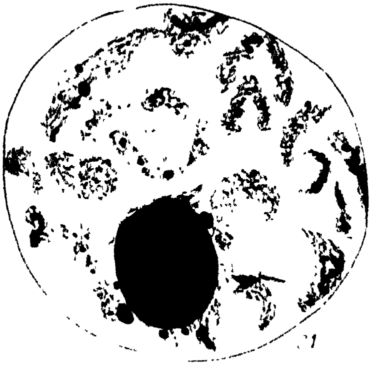
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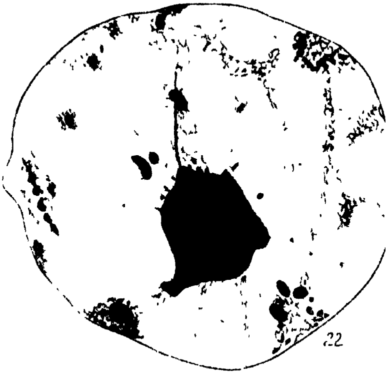
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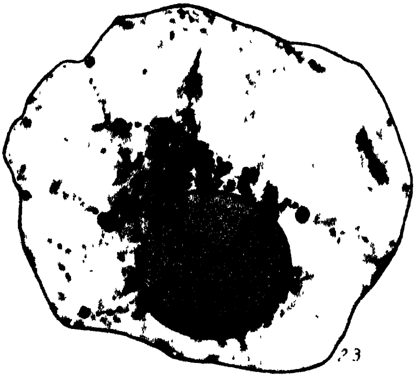
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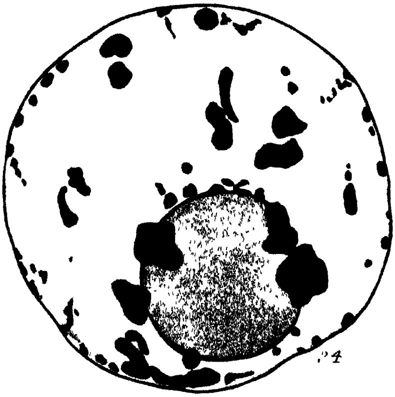
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A STUDY OF THE FUNCTION OF THE EPIDIDYMIS

II. THE IMPORTANCE OF AN AGING PROCESS IN SPERM FOR THE LENGTH OF THE PERIOD DURING WHICH FERTILIZING CAPACITY IS RETAINED BY SPERM ISOLATED IN THE EPIDIDYMIS OF THE GUINEA-PIG

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AUTHOR'S ABSTRACT

Following the isolation of sperm in the epididymides of male guinea pigs by the separation of this organ from the testis, the animals were found to remain fertile for periods varying from twenty to thirty-five days, whether they were allowed to mate but once during this period, or five times during the period, or twenty times during the period. Motile sperm were found as long as fifty nine days after the operation. It was concluded, on the basis of these data, that an aging process which sperm isolated in the epididymis undergo is more important than the number of matings (up to at least nine or ten) in the determination of the period during which sperm motility is retained and in the determination of the period during which fertilizing capacity is retained.

If the behavior of sperm isolated in the epididymides can be taken as an indication of the processes of sperm development which occur normally, the epididymis would seem to be an organ in which sperm may at one time be attaining an optimal functional state and may at another time be aging and becoming incapable of functioning. The possible significance of this suggestion for the rôle of the epididymis in sperm development and for the history of sperm after they leave the testis is discussed.

CONTENTS

Introduction 475
Literature 476
Experimental 480
Discussion 487
Conclusions 489
Bibliography 490

INTRODUCTION

In an earlier paper (Young, '29) the relationship of the epididymis to the development of spermatozoa contained in it was discussed. It was pointed out that the opinions with respect to epididymal function are numerous and varied. They are so diverse, in fact, that it is impossible to construct from the literature any satisfactory conception of the relationship of this organ to its contents, the spermatozoa, or of the changes undergone by sperm from the time they leave the testis until they have reached the posterior end of the genital tract and are in a position to be discharged. There is, therefore, a need for a comprehensive study of what might be called the posttesticular phase of spermatozoan history, and it is this that has been undertaken.

The first experiments (Young, '29) involved an investigation of the basis for the generally accepted opinion that spermatozoa attain full maturity and are strengthened in consequence of some action of the epididymal secretion. No evidence that sperm are strengthened as a result of some specific action of the epididymal secretion was found. On the contrary, the changes which do occur were found to start before the sperm leave the testis—a fact which suggested, 1) that sperm development, not yet complete when these cells leave the testis, continues during their long passage through the epididymis; 2) that the epididymis is essentially a reservoir for sperm in which the processes of sperm development are free to continue after they have left the testis, and, 3) that the stimuli which are responsible for the ripening changes undergone by sperm during this period are the same as those previously effective in the testis.

A second part of the study has involved an investigation of the relationship of the factor of sperm age to the length of the period during which fertilizing capacity is retained by sperm isolated in the epididymis of the guinea-pig; the idea having been expressed by several writers that the epididymis functions essentially to preserve the vitality of sperm during their residence in it.

LITERATURE

The idea that the vitality of mature spermatozoa is preserved during their residence in the epididymis by the secretions of this organ seems first to have been suggested by Van der Stricht ('93). Tournade and Delacarte ('13) drew the same conclusion from an experiment which must have been performed faultily. They observed that sperm placed in connective tissue soon lose all motility. On the other hand, a male rat, whose sperm supply to the epididymis had been cut off by an operation involving ligature of the efferent ducts, impregnated a normal female inhabiting the same cage seven and one-half months after the operation. Later, when the animal was killed and autopsied, motile sperm were removed from the epididymis. Tournade and Delacarte concluded from these observations that sperm vitality can be preserved in the epididymis of the rat for at least seven and one-half months.

Hammond and Asdell ('26) have, more recently, performed a similar operation on the rabbit and made observations on the duration of the retention of fertilizing capacity and motility by the sperm contained in the epididymis at the time of the operation. In the animals they used, however, the sperm were not capable of effecting fertilization after forty days, and no motile sperm were found after the sixtieth day. These experiments, in conjunction with others of Moore and Benoit to be reviewed below, would seem to make it fairly certain that a connection must have been reestablished between the testes and epididymides in the animals used by Tournade and Delacarte.

The most elaborate exposition of the idea that the vitality of sperm is preserved during their residence in the epididymis by some action of its secretion is contained in a series of papers by Braus and Redenz ('24) and Redenz ('24, '25 a and b, and '26). These writers cite evidence from the work of Posner ('05), Rehfisch ('96), and some evidence from their own observations of sperm within the rete tubules of the bull as authority for the conclusion that sperm attain their complete maturity and motility while still in the testis, and move out of the testis into the epididymis by means of their own activity. According to Braus and Redenz, but particularly the latter, sperm vitality would be dissipated very quickly were this activity not limited until the time of sperm discharge. Limitation of sperm activity is accomplished, however, by events occurring within the epididymis, and spermatozoan vitality is thus preserved. It is not clear to the writer whether finally they regard one of three factors as being involved or whether all are considered to be effective. At all events, they attribute the limitation of motion, possibly to the effect of a high carbon-dioxide tension within the epididymis (Braus and Redenz, '24; Redenz, '24 and '26), possibly to the effect of a low oxygen tension (Redenz, '24 and '26), or possibly to the effect of the resorption of electrolytes from the seminal fluid (Redenz, '25 a and b, and '26). According to Redenz, the true seminal fluid which surrounds sperm as they leave the testis is rich in electrolytes, and therefore a medium in which sperm are activated. The electrolyte content becomes reduced, however, as the seminal fluid becomes mixed with the electrolytically poor colloids secreted by the epididymis, and sperm motility is inhibited. The sperm are thought of as remaining non-motile until the time of an ejaculation, when they are thought to be reactivated by the prostatic fluid, considered by Redenz, on the basis of Hirokawa's ('09) work, to be rich in electrolytes.

The theory is too conjectural, and the evidence for the movement of sperm out of the testis by means of their own flagellation is at variance with previously reported observations on the behavior of sperm from different levels of the epididymis and the testis (Hammar, '97, for the dog; Walker, '99, for the dog; Tournade and Regaud, '11, for the rat; Tournade, '13, for the rat, rabbit, guinea-pig, and dog; Rohleder, '24, for man; Mettenleiter, '25, for man and the bull; and Young, '29, for the guinea-pig, rat, ram, and bull).

Another investigator, von Lanz ('26), also regards the epididymis as a sperm reservoir in which the tendency of sperm to move and thus to dissipate their energy is limited by the high hydrogen-ion concentration within this organ. Von Lanz' conclusion was reached after pH determinations had been taken of the testis and epididymis by means of a hydrogen electrode.

The theory that the epididymal secretion preserves the vitality of sperm has been brought to our attention only recently by an entirely new line of work. Benoit ('21, '25, and '26) and Moore ('27 and '28) have shown that the vitality of sperm in the epididymis separated from the testis is preserved only for a limited time. If both testes are separated from the epididymides and removed from the guinea-pig, the sperm isolated in the epididymides become progressively less active upon proper stimulation and lose all traces of motility by the twenty-third day. On the other hand, if both testes are separated from the epididymides, but either one or both are left in the animal, the sperm isolated in the epididymides become progressively less vigorous, but do not lose all traces of the capacity for motility until the sixtieth to the seventieth days following the operation. In either case, the ultimate death of the sperm would seem to be attributable to a gradual loss of vitality. At the same time, the longer retention of the capacity for motion under the condition of the presence of a testis is attributed by both Benoit and Moore to the influence of a hormone elaborated by the testis. Moore and McGee ('28) have even been able to replace this testicular influence in the guinea-pig by the subcutaneous injection of lipoid extract of bull testis.

It is seen, therefore, that the conception of the epididymis as an organ which acts essentially to preserve the vitality of sperm contained in it is not complete. Experiments by Benoit ('21, '25, and '26) and by Moore ('27 and '28), to which those by Hammond and Asdell ('26) should be added, have suggested that any preserving action which the epididymis may have is conditioned by certain external factors: 1) by the life span of the sperm themselves, and, 2) by the presence or absence of the testis hormone.

The experiments reported below are thought to confirm and extend some of these recent ideas as to the factors involved in the preservation of sperm vitality within the epididymis and to add data to those already accumulated on the nature of the changes which occur in sperm during their residence in the epididymis.

EXPERIMENTAL

Following an abdominal incision, the testes and epididymides were withdrawn from the scrotal sacs and the sperm stream from the testes into the epididymides was blocked by the double ligature and severance of the body of the epididymis. The operation does not interfere with the vascularization of the epididymis. Testes and epididymides were then replaced in their normal position in the scrotal sacs. If, later, an adhesion developed which caused the confinement of one or both testes in some part of the abdominal cavity, the animal was discarded, because the higher temperature of this region has been shown to have a destructive influence on spermatozoa contained in the epididymis as well as on immature germ cells contained in the testis (Moore, '28, and Heller, '29).

The animals were divided into three groups on the basis of the frequency with which they were allowed to mate with normal females.

The first group was composed of thirteen males which were allowed to mate at five-day intervals beginning immediately after the operation and continuing until from forty to fifty-five days after the operation. Usually, the animals were killed soon after the completion of the breeding history and the epididymides examined for either motile or non-motile sperm. At this time, in each group of animals, the testes and epididymides were examined macroscopically for the purpose of determining that these organs had been separated completely.

The second group was composed of fourteen males which were allowed to mate at five-day intervals beginning twenty-four to thirty days after the operation and continuing until from forty-five to fifty-five days after the operation. The epididymides were then examined for either motile or non-motile sperm.

The third group was composed of five males, which were allowed to mate as often as they would (about twice every three days), beginning immediately after the operation and continuing until as long as thirty-one days after the operation, following which they were usually allowed to mate at five-day intervals for twenty to thirty days additional. At the end of the breeding history, the epididymides were examined for either motile or non-motile sperm.

The condition of the vaginal closure membrane of each female used in these experiments was examined twice daily between the fifteenth and twentieth days following copulation. If it ruptured, indicating a recurrence of the oestrum (Stockard and Papanicolaou, '19), the mating sixteen to seventeen days previously was considered as having been sterile. If, however, the vaginal closure membrane did not rupture and there was no recurrence of the oestrum, the mating was regarded as having been fertile and the female was isolated until the litter was delivered.

At the end of the experiment, therefore, the breeding history of every male had been recorded in such a way that it could be determined approximately how long the animals had remained fertile, the number of matings during the fertile period, and the number of matings during the subsequent sterile period. In addition, examination of the epididymides when the animals were killed at the conclusion of the experiment provided data as to the condition of sperm still present in the epididymis.

The individual breeding records have been summarized for each of the three groups of males and are presented in tabular form together with some discussion of their significance (table 1).

The males in this group are seen to have remained fertile for from twenty until thirty-five days following the operation. The mean fertile period for the group is 25.6 days. Spermatozoa isolated in the epididymis of the guinea-pig retain their fertilizing capacity, therefore, for at least twenty to thirty-five days following the time of their isolation.

Data contained in the last two columns indicate clearly, 1) that the sperm supply is not nearly exhausted within from six to thirteen matings (the number of discharges was larger) and, 2) that the capacity for effecting fertilization is lost long before the capacity for motility is lost. It was noted, when these data were obtained, that many sperm capable of being stimulated to motility were present in the epididymis for from ten to twenty days after the end of the fertile period.

TABLE 1

Summary of breeding records of males which were allowed to mate at five-day intervals beginning immediately after the testes and epididymides had been separated

ANIMAL NO.	ELAPSED TIME BETWEEN OPERATION AND LAST SUBSEQUENT FERTILE MATING	NUMBER OF MATINGS DURING FERTILE PERIOD	NUMBER OF MATINGS DURING SUBSEQUENT STERILE PERIOD	TIME AFTER OPERATION OF AUTOPSY OF ANIMAL	CONDITION OF SPERM
	Days			*Days*	
114	35	4	2	64	Non-motile
116	29	6	4	50	Motile
117	25	7	6	68	Non motile
118	25	5	4	58	Absent
119	33	7	2	60	Not examined
121	25	6	4	53	Not examined
122	26	5	4	59	Motile
144	22	5	4	53	Motile
145	29	6	3	53	Motile
146	20	4	5	53	Motile
147	25	5	4	53	Motile
158	26	7	3	110	Not examined
159	22	5	4	125	Not examined

The first of the two points noted above is of interest in connection with the relation of the number of copulations to the survival period of sperm. In this experiment sperm contained in the epididymides separated from the testes are seen to have retained their capacity for motility for fifty-nine days following the operation. These data are in close agreement with those from the experiments of Moore ('27 and '28), in which it was shown that guinea-pig sperm may remain alive for as long as sixty to seventy days after such an operation.

The suggestion which follows this surprising result is that the elimination of sperm during copulations, at least within the limits of this experiment, is not a factor of primary importance in reducing their numbers. If the elimination of sperm during copulations were of primary importance in reducing their numbers, one would not expect to obtain the result obtained in this experiment, in which the males mated nine or ten times, to agree so closely with the results obtained by Moore, whose animals did not mate once during the period of observation.

The second point, to the effect that the capacity for effecting fertilization is lost long before the capacity for motility is lost, adds support to the suggestion made elsewhere in this paper that whatever preserving action the epididymis has on sperm is limited by the life span of the sperm themselves. To Moore's ('27 and '28) observation, that sperm become progressively less active following their isolation in the epididymis until finally all traces of motility are lost, and Hammond and Asdell's ('26) observation to the effect that rabbit sperm lose their capacity for effecting fertilization long before they lose their capacity for motion, it is now possible to add the observation that in guinea-pigs sperm likewise lose their capacity for effecting fertilization before they lose their capacity for motion. These three independent demonstrations of the limited life span of sperm isolated in the epididymis would seem to constitute ample evidence for the existence of an aging process in sperm which no action of the epididymis is able to prevent.

Data obtained following observations on the second group of males are given in table 2.

The males in this group are seen to have remained fertile for from less than twenty-four days until thirty-four days following the operation, with the exception of male no. 132. The record of this male is so much at variance with those of other animals that it is believed that the operation was performed imperfectly and that the stream of sperm was not prevented from passing into the epididymides from the testes.

Unfortunately, the animal was killed before the nature of its breeding record was known, and an unusually careful examination of the ducts was not made. In view of the consistent results otherwise obtained, it has seemed permissible to omit this male from consideration with the group as a whole.

TABLE 2

Summary of breeding records of males which were allowed to mate at five-day intervals beginning twenty-five to thirty days after the testes and epididymides had been separated

ANIMAL NO	ELAPSED TIME BETWEEN OPERATION AND LAST SUBSEQUENT FERTILE MATING	NUMBER OF MATINGS DURING FERTILE PERIOD	NUMBER OF MATINGS DURING SUBSEQUENT STERILE PERIOD	TIME AFTER OPERATION OF AUTOPSY OF ANIMAL	CONDITION OF SPERM
	Days			*Days*	
124	24	1	6	63	Non motile
125	Less than 24	0	6	56	Motile
128	Less than 31	0	5	60	Non-motile
129	Less than 30	0	5	57	Motile
130	34	2	2	49	Not examined
131	Less than 25	0	6	54	Motile
132	44	4	1	54	Motile
152	25	1	5	120	Not examined
153	34	2	4	84	Not examined
154	34	2	4	84	Not examined
156	31	3	3	70	Not examined
157	29	2	4	70	Not examined
160	30	1	4	120	Not examined
161	25	1	5	83	Not examined

A mean duration of fertility cannot be determined for the group, because it is not known exactly how long males 125, 128, 129, and 131 remained fertile. It is seen, however, that males in this group remained fertile for from less than twenty-four days until the thirty-fourth day after operation—a period which is not very different from the fertile period of those animals which were allowed to mate four to seven times during the first thirty days after the operation. That the fertile period is somewhat longer in the case of the animals of the second group is believed to be indicated by the greater number of males in the second group that remained fertile thirty days or longer. But, on the whole, the difference is not great.

The fact that the fertile period of a group of males which were not allowed to mate once during the first thirty days after the operation was only slightly longer than the fertile period of a group of males which were allowed to mate four to seven times during this period is surprising. The result can be understood readily enough, however, if primary importance in the determination of the length of the fertile period is attached to the factor of sperm age rather than to the number of copulations. If the number of copulations, rather than sperm age, were the primary factor in the limitation of the fertility of the males in question, one would expect that a male which had not once drawn upon its sperm supply in the epididymis during the first thirty days after the operation would remain fertile much longer than a male which had mated several times during the same period. Actually, however, this did not occur; the difference in the length of the fertile periods was only two or three days, and it was not always even that.

The idea that sperm age is of more fundamental importance than the number of copulations in the determination of the length of the fertile period is also suggested by the point already made (p. 483), that nine or ten matings do not materially reduce the length of the period during which motile sperm can be recovered from the epididymis. If the period during which motile sperm can be found in the isolated epididymis (merely an extension of the period during which fertilizing capacity is retained) is not reduced materially by nine or ten matings, there is no a priori reason why the period during which sperm possess the capacity for fertilization should be reduced by the same number of matings.

It is concluded, therefore, on the basis of the data contained in tables 1 and 2, that the age of sperm is more important than the number of copulations (up to at least nine or ten), first, in the determination of the length of the period during which sperm motility is retained and, secondly, in the determination of the length of the period during which fertilizing capacity is retained.

This suggestion, that an aging process which goes on in sperm is primarily responsible for the limitation of the fertile period of sperm, seems to be strengthened by the results obtained when the duration of the fertile period was determined for those animals which were allowed to mate as frequently as they would beginning immediately after the testes and epididymides had been separated.

TABLE 3

Summary of breeding records of males which were allowed to mate at frequent intervals beginning immediately after the testes and epididymides had been separated

ANIMAL NO.	APSED TIME BE' WEEN OPERATION ANI LAST SUBSEQUENT FI TILE MATING	NUMBER OF MATINGS DURING FERTILE PERIOD	NUMBER OF MATINGS DURING SUBSEQUE STERILE PERIOD	TIME AFTER OPERATI OF AUTOPSY OF ANIMAL	CONDITION OF SPERM
	Days			*Days*	
150	31	19	0	34	Motile
165	24	10	0	28	Motile
166	30	20	6	83	Not examined
167	29	19	5	52	Absent
168	24	14	7	52	Absent

Fewer males are included in this group than in the other two, 1) because the experiment requires a very large colony of non-pregnant females, and, 2) because the results obtained from the five males which were run through the experimental period successfully seemed sufficiently consistent to obviate the establishment of additional experimental animals.

The fact that five males which were allowed to mate from three to twenty times as frequently as those in the other groups remained fertile almost as long as those in the other groups was again surprising. It would seem to confirm the suggestion made above that, in the limitation of the fertile period for sperm isolated in the epididymis, the aging of sperm which has been postulated to occur during the residence of sperm in the epididymis is of greater importance than the number of copulations.

Reference has been made to the observation that the epididymides of males from the first two groups contained many actively motile sperm long after the end of the fertile period. Among the third group of males, on the other hand, the epididymides contained but few sperm even before the end of the fertile period. The factor which limited the duration of the fertile period in males included in the first two groups was not, therefore, exhaustion of the sperm supply, but presumably the age of the sperm which were being discharged.

DISCUSSION

The experiments just described have added to our knowledge of the relationship between the epididymis and its contents, the spermatozoa, and have been suggestive with respect to the relative importance of factors which may be involved in the retention of fertilizing capacity and motility by sperm isolated in the epididymis.

They have, first of all, confirmed the idea which the results of other work (Benoit, '21 and '25); Hammond and Asdell, '26; and Moore, '27 and '28) already suggested, that the epididymis does not function essentially to preserve the vitality of sperm during their residence in it, as Braus and Redenz ('24), Redenz ('24, '25 a and b, and '26), and von Lanz ('26) have claimed, but rather, that sperm may undergo an aging process and finally die regardless of their residence in the epididymis.

This fact, when considered in connection with the results of experiments reported previously (Young, '29), is suggestive of the nature of changes which take place in sperm during their passage through the epididymis. If the behavior of sperm isolated in the epididymis as they were in these experiments can be taken as an indication of the processes of development which occur normally, it would seem that the epididymis is an organ in which the development of spermatozoa, already in progress when these cells enter the epididymis, is free to continue until they have become fully ripened. After that, there is no influence which preserves them in an optimum condition for fertilization if they are not discharged. Their vitality simply becomes lowered and ultimately they become incapable of effecting fertilization and incapable of being stimulated to motion. In other words, the epididymis would seem to be an organ in which sperm may at one time be ripening or maturing, that is attaining an optimal functional state, and may at another time be aging and becoming incapable of functioning. Confirmation of this suggestion is being sought in experiments now in progress.

The experiments just described have been pointed out as suggesting that an aging of sperm which occurs after their isolation in the epididymis may be a more important factor in the loss of fertilizing capacity and motility than the elimination of sperm during copulations. This suggestion leads, in turn, to the further suggestion of the existence of some mechanism whereby sperm are constantly being eliminated from the epididymides and vasa deferentia. The fact that an animal which has not copulated for some time is nevertheless capable of effecting fertilization, is not consistent with the idea that sperm attain an optimal maturity, and then gradually become incapable of functioning, unless the existence of a constant stream of sperm through the epididymis is assumed. Such a stream would be the expression of a mechanism which provides for the elimination of either old sperm or sperm which have attained an optimal condition for effecting fertilization and would become 'old' if they were to remain in the epididymis indefinitely.

This suggestion is not original with the writer. Moore ('28) has also arrived at the conclusion that some mechanism must exist which is constantly accounting for the elimination of excess sperm in the absence of mating. Oslund ('28) has suggested that there is a constant flow of spermatic fluid from the seminiferous tubules through the epididymis and vas deferens into the urethra. His suggestion seems to rest on a hypothetical rather than an experimental basis, however, and it is not linked with the idea that this flow may have the utility of eliminating sperm which have lost or are about to lose their capacity for effecting fertilization. It is from this standpoint that the problem of the existence and nature of such a mechanism is receiving attention as a part of the larger problem of the posttesticular history of spermatozoa.

CONCLUSIONS

1. In the guinea-pig, sperm isolated in the epididymis by the separation of this organ from the testis by operative procedure retain their capacity for fertilization approximately twenty-five to thirty days under three varied conditions: 1) if the males are allowed to mate only once or twice during this period; 2) if they are allowed to mate five to seven times during the period, and, 3) even if they are allowed to mate fifteen to twenty times during the period.

2. In the case of males of the first two groups, motile sperm were recovered from the epididymides of most of the animals examined fifty to fifty-nine days following the operation. This has been taken to indicate that fertilizing capacity is lost long before the capacity for motion is lost.

3. The period of fifty-nine days, during which sperm capable of being stimulated to motion could be found in the epididymides of the animals used in these experiments, was almost as long as the period during which sperm capable of being stimulated to motion can be removed from the isolated epididymides of animals which are not allowed to mate once during the postoperative period.

4. On the basis of the results noted in 1 and 3 above, it is concluded that, up to at least ten copulations, the age of sperm is of more importance than the number of copulations in the determination of the length of the fertile period and in the determination of the length of the period during which the capacity for motion is retained.

5. It is concluded that the epididymis does not function essentially to preserve the vitality of sperm contained in it. It appears, on the contrary, to be an organ in which the development of spermatozoa, which starts while these cells are still contained in the testis, is free to continue until they have become fully ripened. After that, there is no influence which preserves them in an optimum condition for fertilization, and they become 'old,' incapable of effecting fertilization, and, finally, incapable of being stimulated to motion.

6. The fact that 'old' sperm are not known to exist in the epididymis of a normal male suggests the presence of a constant stream of sperm through this organ and the existence of some mechanism which provides for the elimination of 'old' sperm or sperm which would soon become 'old.'

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SOME PHASES IN THE EMBRYONIC DEVELOPMENT OF DIACRISIA VIRGINICA FABR. (LEPIDOPTERA)

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EIGHT PLATES (FIFTY-FOUR FIGURES)[1]

AUTHOR'S ABSTRACT

The cells of the blastoderm which are to form the serosa are two- to four-nucleate, the smaller cells of the embryonic rudiment, uninucleate. The band-like embryonic rudiment encircles the yolk at the equator of the egg The amnion does not begin to form until after the serosa completely covers embryo and yolk. The epithelium of the midgut arises from cells situated at the tips of stomodaeum and proctodaeum. These cells, though not differentiated from adjacent ectoderm at the time of the invagination, are nevertheless interpreted as part of the preprimordium of the endoderm. In the eighty four-hour stage a fold of amnion grows over the dorsal side of the embryo, entirely covering it in the course of the next few hours. A portion of the amnion thus forms the dorsal wall of the embryo. At the completion of the amnion the embryo rotates so that its ventral side is directed toward the egg center. The amnion ruptures just before the larva begins to feed on the yolk which still remains around it The serosa is consumed before hatching, which takes place about five and one-half days after deposition

CONTENTS

Introduction 494
Methods 494
Maturation, cleavage, and formation of the blastoderm 495
The embryonic envelopes and the germ band 498
Growth of the germ band and formation of the lower layer 500
Segmentation of the body, development of the appendages, the differentiation of the lower layer, and the formation of the neural groove, stomodaeum, and proctodaeum 502
The endoderm 507
    Case 1. Gastrulation involving the invagination of the mesodermal and endodermal strips, including the extremities of the latter 511
        a. Enteron derived solely from anterior and posterior cell masses 511
        b. Enteron derived from both anterior and posterior cell masses as well as from the middle strand 511
    Case 2. Gastrulation involving the invagination of mesodermal and endodermal strips, but exclusive of at least the terminal parts of the latter 513
The free cells 514
The stomodaeum, proctodaeum, midgut, and adjacent parts 515
Derivatives of the ectoderm 517
Closure of the dorsal body wall, formation of the heart, completion of the alimentary canal, and revolution of the embryo 520

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Derivatives of the mesoderm 522
The embryonic envelopes 522
Summary 523
Literature cited 524

INTRODUCTION

Experimental work, which is still in progress, on regeneration in insects led to a study of the embryology of Diacrisia virginica, and as a result certain features in its development were observed which seem of sufficient interest to record. Among Lepidoptera, the tineids, pyralids, sphingids, bombycids, noctuids, pierids, and nymphalids have all had their share of attention with regard to certain phases of their developmental history, the silkworm naturally having been a favorite. Though no great attention has heretofore been paid to the maturation stages or to the development and fate of the embryonic envelopes, nevertheless there are enough data available to indicate the great similarity which exists among the members of this order in these particulars.

The species studied, Diacrisia virginica, which is widely distributed over the United States, is quite common in its range. In New York it is found from May to September. Males are frequently taken at light, the females more rarely. The eggs which are laid in clumps upon the host plant hatch in about six days at normal autumn temperature. The eggs upon which this study is based were obtained from females taken in a trap lantern at Ithaca, New York, in August and September by Dr. W. T. M. Forbes. In captivity clumps of eggs are deposited in the evening of five or six successive days until several hundred are laid. The egg masses are held together by a viscid substance which hardens in a short time. Though the chorion is quite thin, nevertheless it seems to be rather impervious to fixing fluids, hence the necessity of piercing it soon after killing in order to permit penetration of the liquid.

METHODS

The eggs were killed in hot (75° to 80°C.) Dietrich's, Bouin's, Gilson's, or Van Leeuwen's fluids and fixed for twelve to twenty-four hours, the chorion having been pierced some time after the fluid had cooled. The material was preserved in 80 per cent alcohol, in some cases having been left for several years before cutting. As the yolk is too brittle to permit sectioning of either the fresh material or that which had been in the 80 per cent alcohol for some time, when embedded in paraffin alone, the clove-oil modification of the paraffin-celloidin method, as described by Kingsbury and Johannsen ('27), was chiefly used. It was found advisable in most cases to remove the chorion in order to facilitate infiltration. After dehydration, the eggs were placed in a mixture of clove oil and absolute alcohol, then into pure clove oil for clearing, and thence for twenty-four hours into a mixture of one part clove oil to two parts of the usual thick ether-alcohol-celloidin mixture. Staining in borax carmine and then destaining the yolk gave good in-toto preparations. Embryos over thirty hours old can be dissected out and freed from most of the yolk. Such preparations are much simpler to orient. Owing to the almost spherical shape of the egg, it is necessary in the earlier stages, in order to orient, to use eggs which have still a bit of the supporting leaf attached. Reconstruction in wax is a useful expedient. The spiral position of the embryo in the egg will prevent in all cases obtaining a series of either longitudinal or cross-sections from a single embryo.

The material was stained on the slides with iron hematoxylin and counterstained with eosin, or erythrosin, or Congo-red. Delafield's hematoxylin was used in some cases.

MATURATION, CLEAVAGE, AND FORMATION OF THE BLASTODERM

The eggs are spherical in form, only very slightly flattened on the lower pole where they are in contact with the surface upon which they are laid. By reflected light they are whitish in color, showing shallow sculpturing. The egg measures 0.75 mm. in diameter. Judging from the constant position of the sperm in the earliest stages, the micropyle is located exactly in the center of the upper side, assuming the surface upon which the egg is deposited to be horizontal. The chorion is quite thin and nearly transparent, but tough and apparently impervious to fixing fluids, hence the necessity of piercing it to insure proper fixation.

The vitelline membrane, thin and structureless, adheres to the yolk, although in some preparations in which the yolk is somewhat shrunken it may adhere to the chorion. No study was made of the fresh yolk. In fixed material there may be distinguished the peripheral layer of formative cytoplasm, over 0.02 mm. thick, and the protoplasmic reticulum with the food yolk in its meshes occupying the center. The peripheral cytoplasm is divided into an outer and an inner zone, the former more eosinophil than the latter (figs. 2, 3), as described by Schwangart ('04) for Endromis, by Huie ('18) for Endemis, and by Eastham ('27) for Pieris.

The egg series studied were fixed at intervals of one hour or less for the first sixteen hours of incubation, then at intervals of about two hours to the fifty-fifth hour of incubation, then at intervals of about five hours until hatching. The long intervals between stages and the rather limited amount of material from the fifty-fifth hour on prevent my giving greater details regarding such questions as the segmentation of the brain, the development of the optic lobes and the innervation of the simple eyes, the origin of the sex cells, the origin of the aorta and its union with the heart, etc. Though the rate of development is more or less dependent upon temperature, for purposes of description it will be convenient to note the time at which changes took place with the material in normally warm autumnal indoor weather.

About forty minutes after deposition, the egg nucleus is to be seen in the anaphase stage of the first maturation division lying a little to one side of the upper pole of the egg and embedded in a mass of the peripheral cytoplasm (fig. 1). At this time, elimination of chromatin takes place in the form of an equatorial disc, the amount eliminated being variable, leaving a constant amount in the daughter cells. This process agrees with Seiler's account ('14) for Lymantria dispar and Orgyia antiqua. As no preparations were found in the metaphase, the exact number of chromosomes was not determined, but in one preparation in the anaphase stage in which the cut was favorably made the number of daughter chromosomes in each half was found slightly to exceed thirty. The chromosomes are oval, more or less uniform in size, estimated to average 0.5 μ in length, thus resembling those of Lymantria dispar as figured by Seiler ('14). The axis of the spindle is perpendicular to the surface of the egg. Centrosome and aster were not noted. The sperm (fig. 5) at this time is located apparently directly under the upper pole about 90 μ from the egg surface. This position seems to be constant, as it was so observed in all the eggs of this stage which were examined. In no case could more than one sperm be found within the egg. The sperm at this time is in the form of a thick, straight rod, in an island of cytoplasm, but without the long rays figured by Henking ('90) for Pieris. In the next two hours the second maturation division has occurred, the female pronucleus has migrated inwardly and fused with the sperm. The first polar body does not divide completely, though a spindle is formed and an irregular division of chromosomes occurs. The second polar body lies in contact with the first.

Since segmentation of the nucleus begins before it reaches the center of the egg, the cleavage cells are located in the upper half of the egg the fourth hour after deposition. Segmentation beginning in the third hour now goes on rapidly, and by the fifth hour a number of cleavage cells are present with polar bodies still in evidence. At eight hours the cleavage cells (figs. 2, 3), dividing mitotically, have nearly reached the surface, a few remaining behind in the yolk to form the yolk cells (fig. 2). The yolk cells increase in number during the next twelve hours, and though some are binucleate, none undergoing mitosis is to be seen. The island of cytoplasm which surrounds the nucleus has its longitudinal axis coinciding with the path of the movement. The cells now penetrate the peripheral cytoplasm (periplasm) to form the blastoderm, the cells destined to form the serosa reaching the surface a little earlier than those which are to form the embryonic rudiment. At this time no sharply marked cell wall can be distinguished. The actively dividing cells (fig. 4) soon cover the entire surface, and by the tenth hour the blastoderm is completed with cell walls distinctly marked. The axis of the spindle of the dividing cells is invariably parallel to the surface of the egg.

THE EMBRYONIC ENVELOPES AND THE GERM BAND

It will now be observed that the cells at the micropylar end and at the lower pole of the egg no longer divide, are larger than the others, and have two to four nuclei in each, while the cells around the sides of the egg (fig. 6) in active mitosis are uninucleate. The former will form the serosa; the latter, the germ band. It should be noted that Ganin ('69), Hatschek ('77), and others long ago figured this condition for the Lepidoptera. Harold (1815) also recognized and figured the serosa, although he did not understand its significance. Kowalevsky ('71) states that the cells which form the germ band in Pterophora are smaller and flatter than those which form the serosa. Woodworth ('89) likewise notes that in Euvanessa the transition between the germ band and the serosa is quite abrupt. A surface view at the junction of germ band and serosa shows a striking difference, the cells of the former being in active mitotic division, while the latter, binucleate and quadrinucleate, have ceased dividing (fig. 6, view from the underside). Eastham ('27) says that in Pieris the line separating serosa and the embryonic rudiment is the seat of rapid cell increase in both parts. In Diacrisia it is seen that cell division occurs throughout the entire rudiment, but not in the serosa. The germ band, when thus first formed, lies wrapped around the equator of the yolk, in length nearly equal to the circumference of the egg, the head and tail ends separated by a narrow isthmus of serosa cells. The band is about one-third as wide as it is long (fig. 8). Though most of the cells of the germ band are actively dividing, the divisions are not synchronous. The serosa cells and those of the germ band are still about equal in thickness. At this stage the serosa is dumbbell-shaped and occupies rather less area than the germ band. With its edges now lapping over the margin of the germ band (fig. 8), the serosa gradually spreads (fig 9), until by the sixteenth hour the germ band and yolk are entirely covered. The increase in the area of the serosa is accomplished by a spreading and thinning, and not by cell proliferation. So far there is no trace of an amnion. The completed serosa at this stage, thinner than the germ band, exhibits at intervals swollen places which mark the position of the nuclei. Grandori ('14) holds that the serosa in the silkworm is completed by the yolk cells which migrate in between the embryo and chorion. This is certainly not the case in Diacrisia.

The belated formation of the amnion may be characteristic for the Lepidoptera, judging from published figures, although Bruce ('87) states that with the bagworm the amnion is formed in the usual manner. It is, however, not clear from his description whether he observed the transitional stages between the beginning of the formation of amniotic folds and the completion of the amnion. Eastham ('27) has given the clearest account in his description of the development of Pieris rapae. In his species it appears that the amniotic fold begins to develop in the manner usual in insects, but later, when only partly formed, the serosa grows on without an accompanying growth of amnion. This type of formation has long ago been observed in the Homoptera as well as in the Lepidoptera (Bobretzky, '78) and other orders. In Diacrisia, as has been said, the amnion does not begin to develop until the serosa wholly covers the surface. At sixteen to seventeen hours, the edge of the germ band becomes slightly reflexed, the edge later (fig. 15, eighteen hours; fig. 16, twenty hours) thins out, expands, and finally, at twenty-eight to thirty hours, completely covers the whole ventral face of the embryo as a very thin membrane, marked here and there with swollen places which indicate the position of the nuclei.

Grandori ('14) maintains that in the silkworm the margin of the amnion is formed from migrating yolk cells, while the middle portion is formed from yolk cells which force their way through the germ band from the yolk. This does not appear to be the case in Diacrisia. The yolk cells at this stage are apparently no more active than before or after the formation of the amnion, nor are actively dividing yolk cells to be observed. Though I am inclined to believe that the entire amnion is formed by a proliferation of the cells which margin the germ band, nevertheless it is possible that only the margin of the amnion is thus formed and its middle portion formed by delamination of cells from the inner face of the serosa or from cells liberated from the ventral (outer) face of the germ band. The fact that proliferating cells are still to be seen on the outer side of the germ band after the amnion has already been formed over it, and that only in one instance in a number of slides has a cell apparently in the process of delamination from the serosa been seen, leads one to question the alternatives. Many cells in mitotic division are to be found still attached to the outer surface of the band (fig. 16) after the twentieth hour, while detached flattened cells, which seem to have arisen from them, may be seen lying between the band and the serosa. In no case, however, has mitosis been observed in these cells after they are detached. It is possible that these detached cells are those which Grandori interprets as yolk cells on an outward migration through the germ band to form the amnion. The difference in structure and size of the nuclei, however, has led me to reject this view.

Eastham ('27) says that for Pieris the separation of amnion and serosa is probably due to progressive delamination of the one from the other. In this case the amnion develops primarily from the edges of the embryonic rudiment and is augmented during later development by additions from the serosa by delamination.

GROWTH OF THE GERM BAND AND FORMATION OF THE LOWER LAYER

While the amnion is forming (eighteen to twenty-eight hours) the embryonic rudiment (germ band) is increasing in size, through active mitosis, forming a cup-like disc with inturned edges. The edges turn into the yolk, the cephalic and caudal ends close over the dorsal (yolk) side, forming an anterior two-lobed, and a posterior simple, pouch. At twenty to twenty-two hours, the yolk material which lies immediately beneath the surface forms yolk spherules, each enclosing one or, less commonly, two or more yolk cells (fig. 16). In the course of the next few hours the entire yolk is reduced to large yolk spherules, each with one or more yolk cells. The nuclei of these cells are distinctly larger than those of the germ band, though smaller than the nuclei of the serosa at this time. Each spherule is surrounded by a delicate yolk membrane. Figure 10 shows a thirty-hour-old embryo viewed slightly obliquely from the dorsal or yolk side. The cradle-shaped embryonic disc, at this stage only about as long as broad, is not bilaterally symmetrical, but shows a slight twist at the caudal end. The cephalic lobes are quite distinct; the body, however, wholly lacks the caterpillar-like appearance which it acquires in the course of the next twenty-four hours when it attains its greatest length (figs. 11 to 13). At thirty-three to thirty-four hours, the turning in of the edges of the disc has reached its greatest development. The head and tail pouches are so deep that a single plane section passed parallel to a plane tangent to the ventral face appears as in figure 14 (a and b). The germ disc now begins to elongate (fig. 11), a median strip eight to ten cells in width (fig. 17) sinks into it. This strip extends from the head lobe longitudinally to the tail, though at first it is not in evidence in either the head or tail pouch, nor does it appear uniformly and simultaneously throughout its length. The growth of the lateral parts of the ectoderm toward the median line and their final fusion result in the median strip becoming the so-called lower layer (fig. 18). The sinking in of the middle strip reduces the width of the embryo, excepting at the head end, which is still two-pouched, and at the other extremity, which is a single pouch which will give rise to the telson. As development progresses, and coincident with the beginning of segmentation of the ectoderm (fig. 12), the lower layer also assumes a more segmental character in that its cells at the intervals between the segments do not multiply as rapidly as in the segments themselves where they form irregular layers (fig. 19, head; fig. 20, neck region; fig. 21, posterior part of abdomen). The mass at the anterior extremity is larger in both width and depth than elsewhere. At no time and at no part of its length is there the slightest resemblance to the formation of a gastrula tube. Neither is there a proliferation at this time along the middle line, as described for Pieris by Eastham. It would appear that the proliferation along the middle line described by Eastham may be the middle (endodermal) strand of the lower layer breaking down, which in Diacrisia occurs later, at the time of the formation of the neural groove, as described below.

At forty-two hours, the two anteriorly directed head pouches, so prominent after thirty hours, are still present, though they have become smaller and shallower. Just posterior to their dividing wall may be seen a mass of cells of the lower layer which in the middle is four or five cells thick (fig. 19). Posteriorly, it is continued as a median lower-layer band (fig. 20). Two hours later, one sees anterior to this mass in the head the lower layer one cell in thickness. Neither stomadaeum nor proctodaeum is as yet in evidence. At this time the gastrula furrow has closed anteriorly (fig. 22), but is not yet obliterated posteriorly by the union of the edges of the ectoderm. The caudal pouch (telson) now has become smaller, though more elongated and tubular, and into it the lower layer extends, apparently without a break.

SEGMENTATION OF THE BODY, DEVELOPMENT OF APPENDAGES, THE DIFFERENTIATION OF THE LOWER LAYER, AND THE FORMATION OF THE NEURAL GROOVE, STOMODAEUM, AND PROCTODAEUM

Until now the lower layer consisted of a longitudinal band of cells, distinctly enlarged at the anterior extremity and to a less degree at the posterior end. We may conceive of it as being composed of two lateral strips which are to form the mesoderm and which will be so designated in the discussion which follows, a middle strand (the Mittelstrang of German authors), and two cell masses, an anterior and a posterior. These two cell masses in Diacrisia I regard as the anterior and posterior extremities of the middle strand, that is endodermic, which later completely break down, the liberated cells being absorbed in the yolk. The extremities of the endoderm (fig. 35, *end*) I consider in this species to be retarded in development, retaining their connection with, and indistinguishable at this time from, the ectoderm, but later to be carried in on the tips of the stomodaeal and proctodaeal invaginations.

Segmentation of the body begins soon after the fortieth hour. The head at forty-four hours consists of a large protocephalon and a three-segmented gnathal region. The protocephalon includes the labrum, which is not yet bilobed, the mouth, the antennae, and the feebly developed postantennae. The prostomium gives rise to the clypeus and front. At this time the antennae are still postoral in position. This is in agreement with Snodgrass' statement ('28) that no true segments can lie morphologically anterior to the mouth. Graber ('90) has specially emphasized this point. The abdomen consists of ten segments and a telson.

Figure 23 shows a section through the head of an embryo forty-five to forty-six hours old. The stomodaeum is now feebly indicated, and opposite it is found the anterior cell mass. There is as yet no trace of the proctodaeum. Paired swellings are now to be observed on the head, thorax, and to a less extent on the abdomen, marking the beginning of antennae, mouth parts, thoracic and abdominal legs. As the appendages lengthen a lumen appears in them. The thoracic legs are now as long as they are broad, the lumen lined with a mesodermal layer. A second longitudinal furrow, the neural groove, begins to form at this time, between the rudiments of the appendages. With its formation, the cells along the median line (the middle strand) are loosened and set free in the body cavity. The fate of these cells will be considered later in the discussion of the endoderm. The mesodermal strips are now separated by the neural groove in the posterior part of the head, in the thorax, and abdomen except at the apex. There is as yet no trace of coelomic sacs, though the lateral margins of the mesoderm are in places dorsally reflexed, the margin thus appearing two-layered for a depth of two or three cells. Toward the caudal end in the last two or three segments is found the posterior cell mass several layers deep and with no longitudinal median interruption. Neither is there any interruption at this stage which can be interpreted as a division between the mesoderm on the sides and the posterior cell mass.

In the part of the head in front of the stomodaeal invagination below the ectoderm a single layer of loosely connected cells may be observed extending into the cephalic lobes. A little more caudad in the region of the stomodaeal invagination is the anterior cell mass (fig. 23) several layers deep. It is not separated nor differentiated from the cells immediately posterior to it. The antennal rudiments have a distinct lumen, the second antennae are feebly indicated. In the region of the mandibles and maxillae the mesoderm extends as a single layer into the evaginations. The mandibles and maxillae are now fully as long as they are broad. Two or three hours later, the anterior cell mass becomes somewhat reduced in size by the setting free of the cells opposite the point of the stomodaeal invagination (fig. 24). By the time the embryo is fifty-two hours old, the anterior cell mass has been forced anteriorly and laterally from the apex of the invagination. Caudad of it there is developed the suboesophageal body (fig. 27, *suboes.b*). In the more generalized Orthoptera (Heymons, Wheeler, '93) this body originates as a paired structure, but in certain Lepidoptera it is single. In Diacrisia it is vaguely bilobed. Hirschler ('27) gives a brief account of the various views concerning its occurrence among insects and of its origin. Wheeler ('93) suggests a possible homology with the so-called green gland of the crustaceans. Heymons holds that it is derived from the mesoderm of the intercalary head segment in Orthoptera—a view shared by Strindberg with regard to the Isoptera. I am, however, inclined to believe with Schwangart, Hirschler, and others that it originates from the endodermal anterior cell mass in the Lepidoptera, although it must be admitted that my material at the critical stages is scarcely sufficient to warrant expressing a positive opinion.

The pre-oral region is lined with a single layer of cells. The tip of the stomodaeal invagination at fifty-seven hours has become quite thin (fig. 29, *m*), the labrum (*lr*) is well developed, and the neuroblasts (*neur*), characterized by their larger size, lie below an irregular layer of mesoderm in the cephalic lobes. At fifty-nine to sixty hours the coelomic sacs, though still quite small and already in evidence several hours before, have reached their fullest development in head segments 4 to 6 (figs. 30, 32, *coel*) with a single pair in the pre-oral region. At this time also the mouth parts have distinctly elongated, though their segmentation is not yet evident (fig. 30, cross-section of the head between mandibles and maxillae; fig. 32, section through maxillary head segment).

Meanwhile segmentation of thorax and abdomen, which began after forty hours, has distinctly progressed. At forty-eight hours (fig. 25, longitudinal section), the mesoderm clearly shows a segmental character. The neural groove is deep and conspicuous, extending from the first gnathal head segment to the last abdominal segment. Figure 26 shows a cross-section through the abdomen of a fifty-two-hour embryo. On each side of the neural groove the neuroblasts are already distinct in each segment at forty-eight hours. Of these there are from two to four pairs in transverse section of the intermediate body segments (fig. 26), though they are not so regularly arranged as described by Wheeler ('89) for the Orthoptera. At fifty hours, daughter cells may be found in mitotic division and more or less continuous longitudinally. Laterad and dorsad is the mesoderm, which in the intermediate abdominal segments now shows well-developed coelomic sacs. Since the lower layer at the beginning consisted of a single layer of cells, and not of a gastrula tube, the coelomic sacs are formed by a folding back of the lateral margin in each segment (fig. 26, *coel*). These sacs, though never large and which have reached their fullest development at sixty hours, are apparent in the protocephalic region, in the gnathal and thoracic segments, and in the abdominal segments, with the exception of those toward the posterior extremity adjacent to the proctodaeum. The thoracic and abdominal legs are now conspicuous evaginations of the body wall and are filled with yolk.

The caudal pouch, so conspicuous and distinctive at thirty hours, becomes smaller and more tubular by the lengthening of the embryo. At forty-eight hours, the tubular part (telson) at the caudal end of the embryo measures about 60 μ in length to the point where it opens to the yolk cavity on the dorsal side. Except on the middorsal line, it is lined with mesoderm. At this time, very near the tip of the caudal pouch on its dorsal (yolk) side, the proctodaeal invagination appears. Mitosis is active in the ectoderm as also in the underlying mesoderm which is carried in by the invagination. A few hours later, the tip of the proctodaeum becomes free from mesodermal cells, but proliferating 'ectodermal' cells appear (fig. 28). With the increase in depth of the proctodaeum the dorsal side of the caudal pouch shortens (fig. 28, *c*). Between the proctodaeum and its envelope (the caudal pouch) there are two layers of mesoderm, which, however, at this stage do not completely cover the dorsal side, except toward the caudal end. The apex of the proctodaeum, as noted above, is now entirely free from mesoderm, though a few free cells remain near it.

At fifty-four hours, the proctodaeum, now about twice as deep as it is wide, shows the first indication of the malpighian tubes in the form of two very short, hollow lobes, latero-ventral in position, each of which in the course of the next two or three hours becomes three-branched. A true basal trunk, however, is not present. The tubes are thick-walled with but few cells in the cross-section (fig. 34, *malp*).

THE ENDODERM

Embryos at fifty-nine hours show no indications of the formation of a midgut epithelium, though soon thereafter the enteron rudiments appear. As interpreted here the earlier-formed endodermal tissue of the lower layer, consisting of the middle strand with its extremities, the anterior and posterior cell masses, has at this time been broken down, while the endodermal rudiments which are to give rise to the midgut epithelium are retarded in development and not yet differentiated from the ectoderm.

The anterior enteron rudiments (endoderm) are first visible at the posterolateral angles of the stomodaeal membrane; the posterior enteron rudiments, at the anterolateral angles of the proctodaeum near the origin of the malpighian tubes. By the sixty-fourth hour these rudiments have elongated ribbon-like, so that the tips of the anterior pair growing backward have nearly or quite reached the tips of the posterior pair growing forward. Cells in mitotic division are to be seen near the tips of these rudiments as well as more remote from the tip. These ribbon-like strands of the endoderm are at first but few cells in width (figs. 45 to 48, *end*). In structure their cells do not differ from those of the stomodaeal membrane, nor is there any indication of an interruption in the continuity of the tissues. This condition is similar to that described by Schwartze ('99) for the lepidopterous genus Lasiocampa and by Toyama ('02) for the silkworm, Bombyx, as well as by a number of workers for species of other orders, and has given rise to the view that the epithelial layer of the midgut arises from the ectoderm. The material available at the critical stages between fifty-nine and sixty-four hours is too meager to permit my giving an extended account of the earliest stage in this development, but to judge from such preparations as are at hand, I cannot but conclude that this tissue arises from cells in the lateral apical angles of the stomodaeal and proctodaeal invaginations. This inference is based on the facts that the tips of the invaginations were already free from the cells of the original lower layer before fifty-nine hours and that the rudiments from which the midgut epithelium arises are continuous with the tissue of the invaginations (figs. 49, 54). A condition such as Eastham ('27) has described for Pieris and as shown in his figures 23, 27, and 28 and by Hirschler ('27) in his figure 70 for Catocala, in which there is a continuity in the tissue at the point where the stomodaeal invagination is to appear, may also be seen in Diacrisia (fig. 19), but this occurs early, before the lower layer has fully severed its connection from the ectoderm.

Selvatico ('81) states that the midgut in Bombyx mori is separated from fore- and hindgut by its own wall, that is, two layers of cells are to be found between the lumen of the midsection of the alimentary canal and the extremities. This is not in agreement with the conditions described by others who have investigated this or other species of Lepidoptera.

The cephalic ends of the lateral parts of the lower layer give rise to the mesoderm of the protocephalon, the lateral mesodermal parts to the muscles of the oesophagus, while the median endodermal part which originally lay where the stomodaeum arises is in part liberated as isolated cells and in part, as I believe, pushed down by the stomodaeal invagination to become the suboesophageal body (fig. 27, *suboesoph.b*), which is thus interpreted as endodermal. In Diacrisia this structure is purely embryonic, as it is no longer to be identified after the end of the fourth day. The cells which lie in the caudal pouch surrounding the proctodaeum give rise to the muscles of the hind intestine.

The question of the origin of the midgut epithelium has long received the attention of embryologists, and its study has resulted in the presentation of conclusions which seem difficult to harmonize. Whether these differences are due to errors in observation or in interpretation or whether the developmental processes differ so greatly in the various insects is not clear. Derivation of the epithelium from the yolk cells, from the mesoderm, from the anterior and posterior mesenteron rudiments, from the middle strand, and from cells migrating from a blastoderm thickening, have all been described. It seems that the several views may be reconciled for most of the pterygote insects, if we assume that the preprimordium of that which I prefer to call the endoderm will give rise to the midgut epithelium, that either all of it may enter into the formation of this tissue or that part may break down and disintegrate, and that in some cases the endoderm may not become wholly differentiated until after the invaginations of stomodaeum and proctodaeum have begun to form after the appearance of the mesoderm. Heider has long ago expressed this idea of the presence of latent endodermal cells which later give rise to the midgut epithelium in Orthoptera and Dermaptera (Heider, Schwangart, '04).

To consider the several cases we may imagine the germ band or disc of a generalized insect as a more or less elongated strip forming a part of the blastoderm (fig. 35). As has already been shown, the serosa which occupies a considerable portion of the blastoderm in Diacrisia is, from the first, morphologically distinct from the remaining cells (fig. 35, *ser*). Between the serosa and the germ band proper there may be conceived to be a zone of cells (*am*), the preprimordium of the amnion, which should perhaps be considered a derivative of the ectoderm. This bounds the germ band, which consists of several parts. First there is a broad zone, the preprimordium of the ectoderm (*ect*), toward the inner margin of which we may suppose to be arranged the preprimordia of the neuroblasts (*neur*). Secondly, within the ectodermal zone there are three longitudinal strips, the median one (*mds*) enlarged at each end (*end*) and two laterals (*mes*). These represent the preprimordia of the endoderm and mesoderm. The preprimordium of the blood cells may be assumed to be located on the margins of the lateral bands. It should be pointed out here that, with the exception of the serosa, the cells of these zones in the material studied are still indistinguishable from each other in the blastula stage. In this scheme the germ cells have not been included, as they are not here considered as germ-layer derivatives.

In the Lepidoptera, as in Diacrisia, in Apis, and many other insects a longitudinal strip of cells is overgrown by the ectoderm, but more typically a gastrula invagination is formed which closes (fig. 39), so that within the endoderm (toward the yolk side) there is now a tube running longitudinally. Figures 36 to 40 illustrate in cross-section for this hypothetical case the successive stages in the formation of the amniotic fold and the gastrula. The inner margins of the ectoderm approach each other, thus bringing the preprimordia of the neuroblasts together on the median line (fig. 39). At the completion of the gastrula tube, we find the whole of the median endodermal strand lying on its dorsal side (fig. 39, *mds*). The formation of the neural groove as a longitudinal ventral invagination of the ectoderm now pushes against the gastrula tube, cleaving it into right and left two-layered strands of mesoderm, the middle dorsal portion of the tube, which, representing the middle strand of the endoderm, breaks down, setting free the cells (fig. 40, *f.end*). The liberated cells from the middle strand may reassemble and give rise to a part of the midgut epithelium. Hirschler ('27) states that in Catocala the cells thus set free from the middle strand attach themselves to the lower side of the yolk, assemble in nests, which then give rise to the middle section of the midgut, and that from a smaller portion of these cells the blood cells arise. Philiptschenko ('12) would term the median longitudinal strand (here called endoderm) 'Mischanlagen,' since he derives the muscles of the midgut and the blood cells as well as the epithelium from this. It appears more probable, however, that muscles and blood cells arise from the mesoderm strips adjacent to the middle strand (*mds*), and not from the strand itself, as shown in figure 35. In addition to the segregation of the mesoderm as two lateral bands, segmentation also occurs, breaking the bands transversely into metameres, each with coelomic cavity (fig. 26, *coel*).

When the lower layer is fully formed, either as a gastrula tube or as a one-layered band, the stomodaeal and proctodaeal invaginations begin. Figures 40 to 44 illustrate the several cases.

Case 1. Gastrulation involving the invagination of the mesodermal and endodermal strips, including the extremities of the latter

In the event of the formation of a complete tube (figs. 39 to 42) or when only a band is formed (figs. 43, 44), the anterior and posterior enteron rudiments lie at the extremities of the gastrula invagination. The stomodaeal and proctodaeal invaginations now impinge upon these rudiments, pushing them into the yolk (figs. 42 to 44). As described by a number of authors for numerous species of insects belonging to six principal orders, these rudiments each give rise to two strands, those from the stomodaeum growing backward, those from the proctodaeum growing forward, until they meet in the middle. The strands also increase in width, spreading out above and below until they fuse along the median longitudinal lines surrounding the yolk and thus completing the midgut epithelium. Instead of lateral strands being formed, in some insects the rudiments develop cup shape, the edges of the cup-like stomodaeal enteron rudiment gradually approaching those of the proctodaeal enteron rudiment until fusion occurs, completing the tube. Two modifications may be considered under this case:

a. Enteron derived solely from the anterior and posterior cell masses. In this case the middle strand (fig. 35) may be conceived as being vestigial or wanting or as having broken down, the cells being destroyed in the yolk. This occurs in Apis, Calliphora, Chironomus, Gryllotalpa, Blatta, Leptinotarsa, Platygaster, Pyrrhocoris, Endromis, and Zygaema (Schwangart, '04), Pieris (Eastham, '27).

b. Enteron derived from both anterior and posterior cell masses as well as from the middle strand. Differs from *a* in that the cells of the middle strand contribute to the formation of the enteron. This occurs in Periplaneta, Phyllodromia, Donacia, Gasteroidea, Hydrophilus, and Catocala (Hirschler, '06).

Here all the cells on the median line are liberated when the neural groove forms. Cells on the splanchnic (inner) layer of the tube then take part in the formation of the enteron as in Hydrophilus (Cholodkowsky, '91). Cells on the outer side of the tube, which are mesodermal, may in part form blood cells and in part perhaps be destroyed in the yolk. Where a one-layered band is invaginated, as in Catocala, the middle strand contributes to the formation of the enteron.

Some of the earlier writers, mainly prior to 1900, have ascribed the origin of the enteron to the yolk cells, Tichomiroff, '79, '92). In some cases at least it is evident that during the development these cells are augmented in number by cells set free from the middle strand of the lower layer and from which the enteron may arise. In other instances some of these earlier investigators maintain that the enteron is derived from the splanchnic layer of the 'mesoderm' by delamination. It seems probable, however, that here also the cells concerned arise from the middle longitudinal section of the lower layer, that is, from the middle strand, which is endodermal (fig. 35, *mds*).

Quite recently, Leuzinger and Wiesmann ('25) ascribe the origin of the epithelium of the midgut of the walking-stick (Carausius morosus) to cells which are set free from the mesal margins and from the mesal processes of the segmentally arranged coelomic sacs. These cells migrating into the yolk-cell membrane (Dotterzellmembran) here replace the yolk cells, and from this modified membrane the enteron is derived. The explanation here appears to be that the middle endodermal strand is divided longitudinally, the halves first remaining attached to the lateral mesodermal bands; then, breaking down into free cells, later form the midgut epithelium.

Case 2. Gastrulation involving the invagination of mesodermal and endodermal strips, but exclusive of at least the terminal parts of the latter

The ends of the endodermal strip thus left behind are retarded in their development until after they are carried in on the tips of the stomodaeal and proctodaeal invaginations. In this case we may assume that the gastrula invagination includes both mesoderm and endoderm, but exclusive of the ends of the latter (fig. 35, *end*). That is to say, these endoderm tips are not included in the formation of the lower layer, but that a bit of the endodermal preprimordium is still left with the ectoderm at the points where the stomodaeal and proctodaeal invaginations are to occur. The lower layer (either a flattened tube or a single layer) thus includes the two lateral mesodermic bands, and the endoderm lacking the extremities. The parts of the endodermal preprimordium which remained continuous with, and indistinguishable from, the ectoderm are now raised up at the tips of the anterior and posterior invaginations, develop into the rudiments of the midgut epithelium, producing the appearance of an ectodermal proliferation as described for insects of several orders by a number of authors, as well as for Diacrisia by the present writer. From these rudiments the epithelium of the midgut develops as described under the first case. This type of development has been described, though differently interpreted, for some Orthoptera, Coleoptera, Diptera, Odonata, and Homoptera as well as for the lepidopterous genera

Bombyx (Toyama, '02) and Lasiocampa (Schwartze, '99). Wheeler ('93) observes that in Telea polyphemus many cells pass into the yolk from the anterior and posterior cell masses, which leads one to believe that this species also belongs to this group. Strindberg ('15) states that in Bombyx the anterior part of the midgut epithelium arises from the anterior cell mass. This is at variance with Toyama's account.

It should be pointed out that the lower layer in Diacrisia does not sink below the level of the ectoderm simultaneously and uniformly throughout its length. In view of this, it is also quite conceivable that the anterior and posterior extremities may lag behind in some cases as suggested above, thus offering an explanation as to how in pterygote insects the midgut epithelium may arise from the endoderm alone and thus reconcile the observations of many investigators.

THE FREE CELLS

These free cells in Diacrisia are of six sorts: 1) The yolk cells that remain behind in the yolk at the time of the outward migration of the cleavage cells to form the blastoderm have already been mentioned. They later occupy the center of the yolk spherules and finally disintegrate. 2) The migratory cells from the ectoderm in the early stages of development, some of them probably equivalent to the paracytes of earlier writers, and which also degenerate. 3) With the formation of the neural groove the ectoderm pushes up against the median line of the lower layer, setting free the cells of the middle strand of the endoderm. These may, no doubt, assemble to assist in building up the midgut epithelium in some insects as described by Hirschler for Catocala, but in Diacrisia they appear to degenerate in the yolk after the sixty-hour stage. 4) When the stomodaeum and proctodaeum are forming, the cells from the anterior and posterior cell masses are set free. In my interpretation these cell masses in Diacrisia are also endodermal, representing the extremities of the middle strand. The endoderm rudiments (enlarged ends distad of the middle strand), or at least their apical portions, I consider to be retarded in their development, as has already been noted. 5) At the time of the formation of the neural groove, cells are liberated along the mesal margins of the mesodermal strands (fig. 35, *mes*). The cells thus liberated from the strands I consider to be blood cells, some of them perhaps disintegrating in the yolk. The blood cells may be found in all subsequent stages in the development. They are found in the body cavity and in small numbers in the large epineural sinus. They are especially numerous along the margins of the mesoderm after the sixty-fifth hour, and later, after the ninety-fifth hour, are to be found in the heart also. The nuclei of the blood cells resemble those of the adjacent mesoderm. 6) Some cells are also set free from the suboesophageal body and are to be found near the body from the sixty-five-hour stage and later. They may be distinguished from the blood cells by their larger size and greater amount of vacuolated cytoplasm. Toyama ('02) interprets these as blood cells.

THE STOMODAEUM, PROCTODAEUM, MIDGUT, AND ADJACENT PARTS

As has been said, the stomodaeum is feebly indicated at forty-five hours, while, two or three hours later, there are traces of the proctodaeum. The latter grows rather more rapidly, so that at fifty-two hours it is larger than the stomodaeum (figs. 27, 28). The lumen of the stomodaeum becomes slender and the membrane at its extremity quite thin. Figure 33 represents this structure at sixty-five hours. It is now about 0.10 mm. in length, projecting inward nearly at right angles to the axis of the body. Dorsally and laterally, it is surrounded by the mesoderm from which the muscle layers are developed; ventrally, there lies the unpaired suboesophageal body. At this stage the proctodaeum (fig. 31) is as long as the stomodaeum, but with larger lumen. The malpighian tubes arise at its tip, three on each side, and as they develop they extend parallel and caudad for about three-fourths of its length. Though originating from a pair of invaginations in the same manner as described by Schwartze

('99) for Lasiocampa, the short basal stem is not evident in Diacrisia. The splanchnic or visceral layers of the mesoderm from which the muscles of the midgut are formed are now visible as two longitudinal bands of cells extending from the labial head segment caudad to the origin of the malpighian tubes. The endodermal strands, which arise from the lateroposterior angles of the stomodaeum, lie dorsad of the splanchnic mesodermal strands. The cells of the latter are arranged transversely. Figure 34 represents a somewhat obliquely cut longitudinal section. Figures 45, 46, and 47 show cross-sections of these bands (*spl.m, mes*).

At eighty-two hours, the closing membrane of the stomodaeum has spread out cup-shaped (figs. 50, 54). The continuity of this membrane with the endoderm is clearly indicated from the sixty-fifth hour onward (figs. 49, 54). Although at sixty hours not yet apparent, at sixty-five the proctodaeum shows the six longitudinal folds so characteristic in many groups of insects. The splanchnic layer of the mesoderm and the endoderm strands soon broaden, growing around the yolk. The endoderm (midgut epithelium) fuses at eighty-four hours ventrad of the yolk (fig. 53, *end*), a little later the mesoderm also fuses. Shortly after the embryo rotates on its longitudinal axis (at eighty-nine hours), both these layers have met on the dorsal side also. The fat-body developing from the somatic mesoderm is composed of cells which are nearly as large as the oenocytes, but differ in being vacuolate. It is not until during the course of the fourth day that fat and muscle cells are sharply differentiated, the latter becoming striate a few hours before emergence of the larva.

Of the origin or early development of the gonads nothing was discovered. Toward the end of the third day they are already well advanced, the genital ridges being formed laterally on the inner somatic mesoderm of the coelomic sacs. At seventy-seven hours, they may be found about 20 μ cephalad of the tip of the proctodaeum. Figure 53, which represents an eighty-four-hour-old embryo, shows them on line with the dorsal edges of the endoderm (fig. 53, *g*) in the fifth abdominal segment. Rizzi ('12) describes a group of large cells which appear near the caudal end of the germ band in the silkworm at twenty-four hours and which he interprets as the sex cells. Later in the course of development, they diminish in size. As they were not followed through to a later recognizable stage, their character is not certain. No such structures were observed in the earlier stages of Diacrisia.

DERIVATIVES OF THE ECTODERM

The neural furrow is already well indicated at forty-five hours. At forty-eight hours, neuroblasts, already referred to, are distinctly differentiated from adjacent cells of ectoderm in head and body segments. In its earlier stages the part of the head exclusive of the gnathal region is not segmented. The supra-oesophageal ganglion formed by delamination from the ectoderm of the cephalic lobes at sixty-five hours shows by the presence of its neuropile a distinct tritocerebrum, which is further identified by its commissure. The deutocerebrum is obscured by the great development of the protocerebrum. Before the end of the third day the divisions of the suboesophageal ganglion are well developed, each clearly showing the fibrillar character of the anterior and posterior neuropile masses. In the ganglia of the thorax and abdomen this division of the neuropile in each segment is also already well indicated at this time. At sixty-five hours, ganglia are distinct in all abdominal segments, including the tenth. As development progresses the nerve cord separates from the adjoining ectoderm. At sixty-five hours it is already quite free, except along the median furrow where ectoderm and cord merge. Six hours later, the edges of the ectoderm have fused along the middle line.

Schwartze ('99) finds that in Lasiocampa the visceral sympathetic nervous system originates as evaginations on the dorsal side of the stomodaeum. The critical stages are lacking in my material. These ganglia are not yet in evidence at fifty-nine hours, but at sixty-five hours they are already in position on the middorsal line above the stomodaeum. Twelve hours later, the frontal ganglion may be observed. Figure 51 represents a longitudinal section through the suboesophageal and first thoracic ganglia of an embryo eighty-two hours old. The simple eyes, of which five pair develop, arise from clusters of moderately large ectodermal cells, which appear early in the fifth day. Their subsequent development was not followed.

Shortly before the endoderm begins to develop, apodemes appear in the head. The first, near the base of each mandible, a deep tubular invagination extending caudad, is one of the anterior arms of the tentorium. Two other shallower invaginations for the attachment of the mandibular muscles appear in this head segment. A larger apodeme on the side of the maxilla extending back into the labial head segment forms one of the posterior arms of the tentorium. In the second maxillary segment there are two pairs of invaginations; the inner pair forms the silk glands, the outer pair develops into the so-called hypostigmatic gland of Toyama ('02). The invaginations for the silk glands (fig. 45, *silk g*) are found just laterad of the neural groove in the labial segment. By the sixty-fifth hour they extend back as simple, single-layered tubes to opposite the second pair of thoracic legs; by the eighty-fourth hour they extend beyond the seventh abdominal segment.

The 'hypostigmatic gland' (Toyama, '02) develops as an invagination near the root of the second maxilla (labium). At sixty-five hours, the invaginated cells are larger than those adjacent, the entire invagination spherical in form and gland-like in appearance (fig. 45, *gl*), with the mouth of the invagination directed cephalad. During the course of the next ten hours the structure has migrated mesad and slightly caudad, coming to lie in front of the prothoracic legs, and has lost its attachment with the surface. Later, its cells can no longer be distinguished from those of the adjacent mesoderm. Nelson ('15) suggests that this structure may represent the corpora allata, though I am more inclined to consider it the rudimentary cervical gland which is so highly developed in the caterpillars of Schizura concinna, Dicranura vinula, and other notodontids, though vestigial in many other lepidopterous larvae. In the silkworm (Toyama, '02) it persists in the larva as branched glands in the first thoracic segment.

In lepidopterous larvae the first thoracic segment bears a distinct spiracle, and in some cases a vestigial one may be demonstrated between segments 2 and 3. In the embryo at sixty-five hours on the anterior margins of thoracic segments 2 and 3 the invaginations for the tracheae have advanced sufficiently to permit the recognition of a short anterior and a posterior branch. Their walls are single-layered and indistinguishable from the adjacent ectoderm (fig. 47, *tr inv*). There is no indication of a tracheal invagination on the prothorax. Invaginations for the abdominal tracheae are equally advanced at this stage, located on the anterior part of segments 1 to 8, the last one being the largest. The ninth and tenth segments, as well as the telson, are not provided with tracheal invaginations. By the seventy-seventh hour, the most anterior spiracle has migrated onto the prothorax, while the second one is no longer apparent.

The oenocytes, of ectodermal origin and associated with the tracheal invaginations, lie in clusters ventrad of the spiracles. In the eighty-four-hour stage they resemble the fat-cells in size, but are not vacuolate; consequently, they stain more deeply. They are conspicuous in the newly hatched larva.

The trichogen cells are by far the largest and most conspicuous ectodermal cells in the embryo. They cannot well be confused with the oenocytes, being larger and in this species are more dorsad in position. At fifty-nine hours they are not yet differentiated, but at sixty-five hours they may be found in both thoracic and abdominal segments (fig. 47, *trich*). They are so large that they extend far below the level of the neighboring ectodermal cells. In some sections, as in the one figured, they are cut tangentially, so that here they appear among the underlying mesodermal cells.

Tichomiroff ('78) has long ago suggested that these large cells are 'hair cells,' that is, trichogens. The seta develops shortly before hatching. At this time both oenocytes and fat-cells may be seen adjacent to the trichogen cells and are very easily differentiated.

Shortly after the beginning of body segmentation, the rudiments of the appendages appear. At forty-five hours the mouth parts and the thoracic legs are as long as broad, with the lumen distinctly marked. At sixty-five hours, mouth parts and thoracic legs show distinct segmentation. Twelve hours later, thoracic legs are three-segmented; the maxillae and labium, two-segmented. The labrum, at first simple, at sixty-five hours becomes bilobed. The abdominal prolegs, which at forty-two hours are not distinguishable from the rudiments on abdominal segments 1, 2, 7, 8, and 9, soon develop rapidly, while the rudiments on these segments become reduced.

CLOSURE OF THE DORSAL BODY WALL, FORMATION OF THE HEART, COMPLETION OF THE ALIMENTARY CANAL, AND REVOLUTION OF THE EMBRYO

The endoderm strands have fused on the longitudinal mid-ventral line at eighty-four hours, but dorsally the edges are still far apart (fig. 53, *end*). The middle portion of the dorsal body wall is likewise still open (umbilical passage), although at the ends the amnion has already closed over the dorsum (figs. 50, 52), the anterior fold having extended as far back as the tip of the stomodaeum; the posterior fold, as far forward as the base of the malpighian tubes. Five hours later, the edges of the amniotic folds, which at eighty-two hours (fig. 50, *am.f*) were still far apart, have fused, completing the dorsal wall.

The cardioblasts arise on the lateral margins of the somatic mesoderm. At about ninety-two hours, they are to be seen in cross-section as a small group of cells arranged crescent-like with the convex side directed laterad, and blood cells lying between the horns. Posteriorly, in the region of the hind intestine, the horns of the crescentic group of cardioblasts have fused to complete the heart. Two or three hours later, the heart and aorta are also completed anteriorly, while the edges of the splanchnic mesodermal layer likewise meet dorsally, completing the midgut. Blood cells are abundant in the heart. Communication between the midgut and the stomodaeum and proctodaeum is established at this time by the disappearance of the stomodaeal membrane and the tip of the proctodaeum. The closure of the dorsal wall of the midgut at ninety-four to ninety-five hours results in the inclusion of yolk. From about the thirtieth hour, when the embryo begins to sink into the yolk, until the rupture of the amnion a few hours before emergence of the larva, the embryo is immersed, the peripheral yolk being about the thickness of the diameter of one yolk spherule during the greater part of this time (fig. 135, Korschelt and Heider). This peripheral yolk which is present at the time of the rupture of the amnion is soon consumed by the young larva a few hours before the rupture of the chorion. At first, after the completion of the dorsal wall of the midgut, there are still yolk cells to be found, both in the peripheral yolk and that contained in the alimentary canal, but later both yolk masses are liquefied and the cells are no longer present. No yolk is to be found in the oesophagus or in the hind intestine.

When the dorsal body wall is closed the embryo rotates about its longitudinal axis until the ventral surface is directed toward the center of the egg. In the material studied the rotation was about half completed at eighty-nine hours. Figure 50 shows a longitudinal section of an eighty-two-hour-old embryo. Here the amniotic fold has already extended beyond the tips of the stomodaeum and proctodaeum. In the figure the proctodaeum has been cut somewhat obliquely, owing to the slightly spiral position of the embryo in the egg. A cross-section through the proctodaeum of an embryo of the same age is shown in figure 52, showing how the inner fold of the amnion forms the dorsal wall—a condition also to be found in Meloe among the Coleoptera. This dorsal wall later becomes somewhat wrinkled at the posterior end of the embryo, which may indicate a vestigial second dorsal organ. After the closure of the umbilical passage and the dorsal wall of the midgut and the breaking down of the stomodaeal membrane, the oesophagus telescopes a short distance into the midgut, forming the oesophageal valve.

DERIVATIVES OF THE MESODERM

As here interpreted, all parts in this insect derived from the lower layer belong to the mesoderm. The middle strand of endoderm has broken down and has been destroyed in the yolk. In the foregoing account the origin of muscles, fat, heart, blood, and genital ridges, that is, mesoderm derivatives, have all been briefly discussed. The suboesophageal body, an embryonic structure in Diacrisia, and identified by some as mesodermal, I am inclined to consider of endodermal origin.

THE EMBRYONIC ENVELOPES

As has previously been stated, the cells which are to form the serosa are already sharply differentiated in the blastula stage ten hours after the deposition of the egg. The serosa gradually spreads over the embryonic rudiment, entirely covering the surface by the sixteenth hour. The amnion which does not begin to form until the serosa is quite complete, that is, at sixteen to seventeen hours, entirely covers the ventral face of the embryo twelve to fourteen hours later. From then on, with the increasing width of the embryo the amnion spreads by stretching rather than by cell division until it wholly covers the embryo with the closure of the dorsal wall at about eighty-seven hours. A small portion of the amnion formed from its inner fold (figs. 50, 52, *am.f*) completes the dorsal wall of the embryo, the remainder forms a closed envelope around the embryo. Between this envelope and the serosa there still remains a considerable quantity of yolk. A period of over twenty-four hours elapses after the completion of the amnion as a closed envelope, during which period the alimentary canal closes dorsally and the heart is completed. The small part of the amnion which forms the dorsal wall of the embryo is apparently transformed into the dorsal hypodermis without the formation of the so-called 'second dorsal organ.' Some time after 112 hours, the amnion is ruptured and the larva begins feeding upon it and upon the yolk material which is still present between the amnion and serosa. At 132 hours, or five and one-half days after the deposition of the egg, the larva, having wholly consumed the remaining yolk and the serosa also, liberates itself by rupturing the vitelline membrane and the chorion.

SUMMARY

The cells of the blastoderm which are destined to form the serosa are two- to four-nucleate and larger than the uninucleate cells of the embryonic rudiment. The embryonic rudiment, which is band-like at first, encircles the yolk at the equator of the egg. The serosa gradually spreads over the embryo, wholly covering it and the yolk, thus forming an envelope which lies immediately below the vitelline membrane. Upon completion of the serosa, the amnion begins to develop, covering the ventral face of the embryo twelve to fourteen hours later. The young embryo, which first appears as a broad band, later becomes as broad as it is long, disc-like, with inturned edges, later still elongating at the time segmentation begins. A gastrula tube is not formed, but instead, a band somewhat similar to that in the embryo of the honeybee. The epithelium of the midgut arises from cells situated at the tips of stomodaeum and proctodaeum. These cells at the time of the invagination, though not differentiated from the adjacent ectoderm, are nevertheless interpreted as part of the preprimordium of the endoderm. This view is taken, since it has been shown that the lower layer does not develop uniformly and simultaneously in its entire length, and the anterior and posterior cell masses break down entirely before the enteron rudiments appear. At eighty-four hours after the deposition of the egg, a fold of the amnion grows over the dorsal side of the embryo, entirely closing it in the course of the next few hours. A small portion of the amnion thus

forms the dorsal wall of the embryo, while the greater part forms an embryonic envelope. The embryo lies curled up with the ventral side outermost, until the amnion completely covers it, then rolls over on its longitudinal axis so as to lie with dorsal side directed outwardly. Such yolk as still remains outside of the body at the time of the closure of the dorsal umbilical passage is consumed by the larva before its emergence, which takes place at about five and one-half days. The amnion ruptures when the larva begins to feed upon the yolk, the serosa is consumed just before hatching.

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ABBREVIATIONS

am, amnion
am.f, amniotic fold
ant, antenna
bl, blood cells
coel, coelome
ect, ectoderm
end, endoderm
f, fat-cells
f.end, free endodermal cells
g, gonad
gl, hypostigmatic gland
l, longitudinal muscle layer
lab, labium
lg, leg
ll, lower layer
lr, labrum
malp, malpighian tubes
max, maxilla
md, mandible
mes, mesoderm
mds, middle strand of the endoderm
mus, muscle layer
n.c, nerve cord
neur, neuroblasts
neur.g, neural groove
oen, oenocytes
p.lg, proleg
proct, proctodaeum
sal.g, salivary gland
ser, serosa
silk g, silk gland
silk g.inv, silk-gland invagination
som.m, somatic layer of mesoderm
sp, sperm
spl.m, splanchnic layer of mesoderm
stom, stomodaeum
suboes.b, suboesophageal body
suboesoph.g, suboesophageal ganglion
tel, telson
tr, trachea
trich, trichogen cells
tr.inv, tracheal invagination
x, transverse muscle layer
y, yolk
y.gl, yolk globules (spherules)

PLATE 1

EXPLANATION OF FIGURES

1 First maturation division of egg nucleus, showing the disc of eliminated chromatin material, forty minutes after the egg is laid. × 1150.

2 Segmentation nuclei nearing the surface. An island of cytoplasm surrounds each nucleus. Vitellophags left behind in the outward migration of the nuclei. Embryo eight hours old. × 200.

3 As in figure 2. Eight hours old. × 500.

4 Blastoderm nearing completion. Cell wall not yet in evidence. Chorion and vitelline membrane not shown. × 1000.

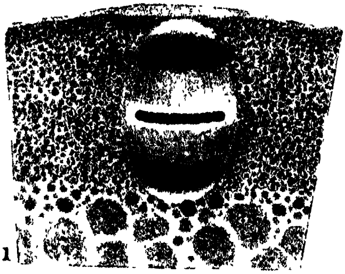
5 The sperm (*sp*) at the time of the first maturation division of the female nucleus. × 300.

6 View of fragment of the blastoderm as seen from within, at the junction of germ band and serosa, about ten hours after deposition of the egg. Diagrammatic.

7 Cross-section of same, showing beginning of the overgrowth of the serosa. × 500.

EMBRYONIC DEVELOPMENT IN DIACRISIA

O. A. JOHANNSEN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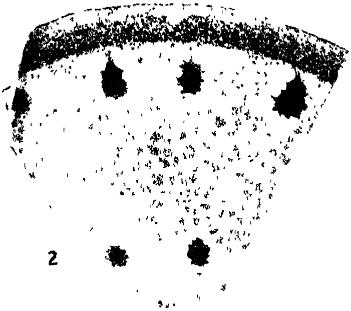
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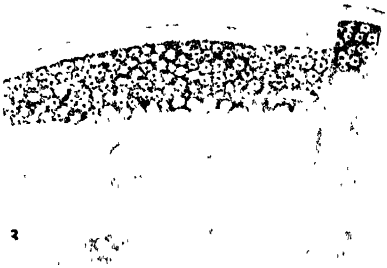
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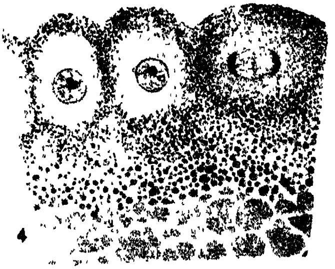
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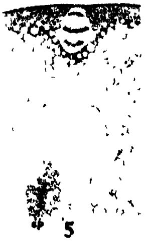
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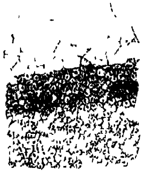
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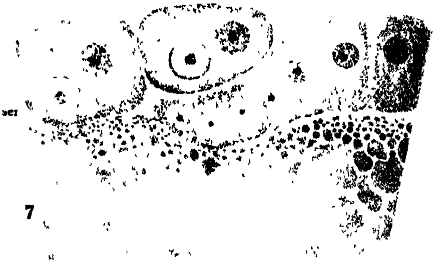

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PLATE 2

EXPLANATION OF FIGURES

8 Surface view of the embryo, ten hours old. The smaller cells forming the germ band are in active mitosis. The larger binucleate and quadrinucleate cells forming the serosa have ceased dividing. Chorion removed. × 75.

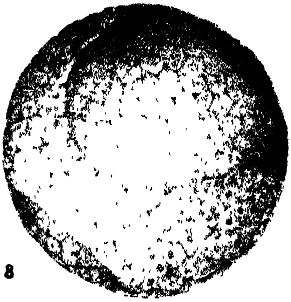
9 Section of germ band lateral in position with the serosa beginning to spread over it. The space between yolk and the cellular layer is due to shrinkage in fixation. Fourteen hours old. × 70.

10 Embryo free from yolk and envelopes, as seen obliquely from the yolk side. Thirty hours old. × 100.

11 Same. Thirty-five hours old. × 100.

12 Same. Forty-two hours old. × 100.

13 Same. Fifty-four hours old. × 140.



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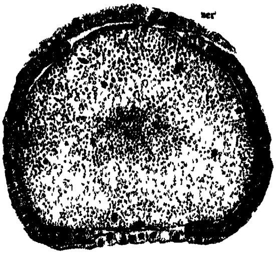

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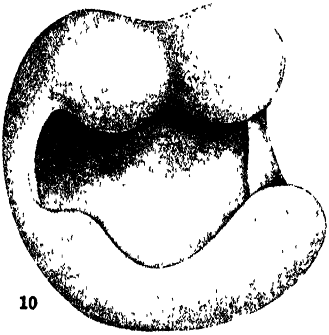

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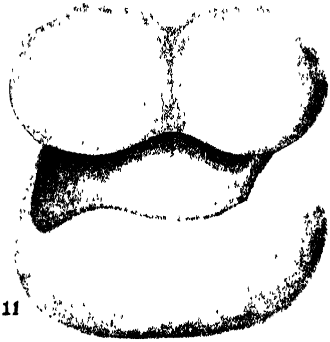

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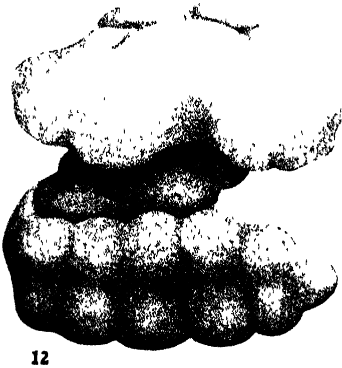

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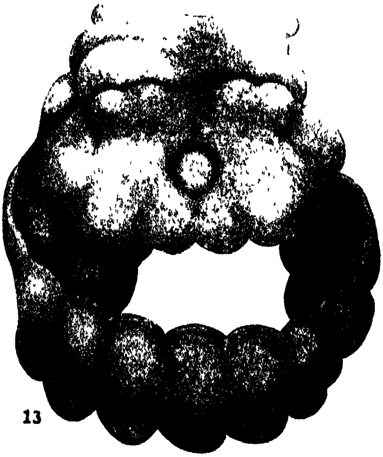

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PLATE 3

EXPLANATION OF FIGURES

14 Plane section parallel to a plane tangent to the ventral face. *a*) Section through the head lobes. *b*) Section through the caudal pouch. Thirty-two hours old. × 100.

15 Amnion beginning to form. Eighteen hours old. × 160.

16 Section of egg when amnion is beginning to form. Yolk beginning to form yolk spherules. Twenty hours old. × 90.

17 Section through abdomen of thirty-four-hour embryo, with differentiation of lower layer. × 175.

18 Section of anterior part of abdomen of thirty-five-hour embryo. × 190.

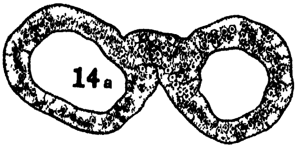
19 Cross-section through the head with gastrula groove, of forty-two-hour embryo. × 190.

20 Section through neck region of forty-two-hour embryo. × 190.

21 Section through posterior part of abdomen of forty-two-hour embryo. × 150.

22 Section through the head of forty-four-hour embryo. × 175.

23 Same, forty-five-to-forty-six-hour embryo. × 175.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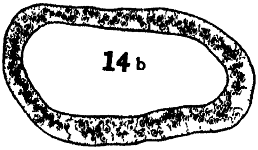
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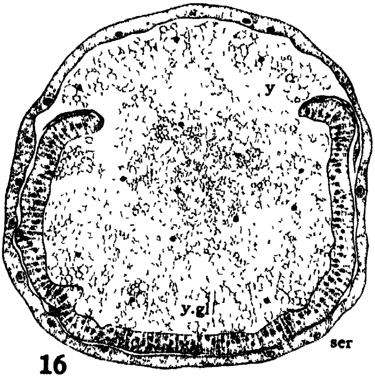

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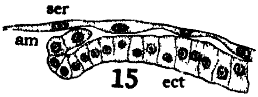

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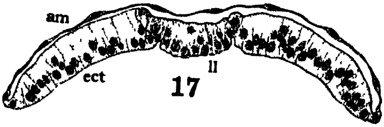

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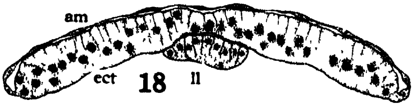

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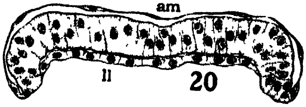

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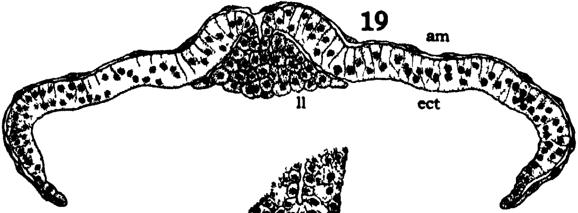

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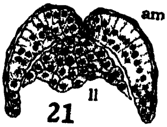

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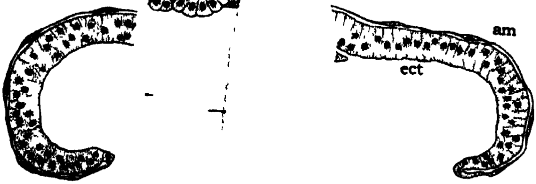

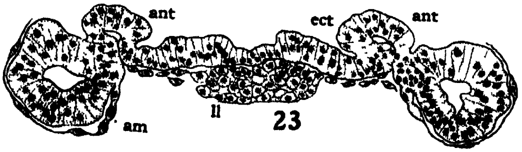

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PLATE 4

EXPLANATION OF FIGURES

24 Section through the head in the region of the stomodaeum. × 200.

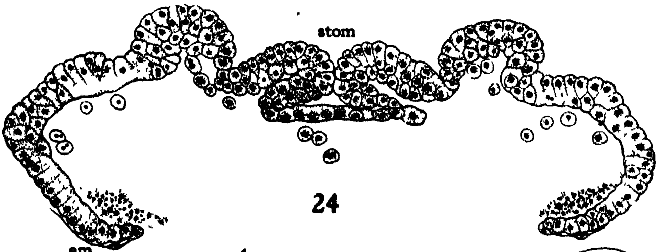
25 Longitudinal oblique section. Such a section may be obtained from an embryo forty-eight hours old by making a slightly oblique frontal cut, entering the right-hand frontal lobe (fig. 12). The proctodaeum and telson are cut off in the section. The Roman numerals indicate abdominal segments. × 150.

26 Cross-section through abdomen of fifty-two-hour embryo. × 275.

27 Median longitudinal section of head of fifty-two-hour embryo. × 290.

28 Median longitudinal section of proctodaeum. Remains of caudal pouch at *c*. Embryo fifty-two hours old. × 290.

29 Cross-section of head through stomodaeum at fifty-seven hours. × 200.



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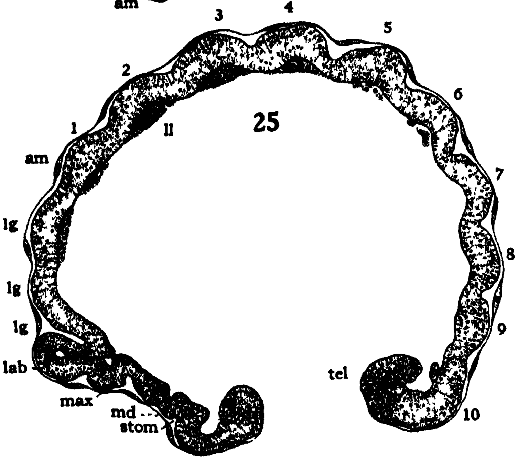

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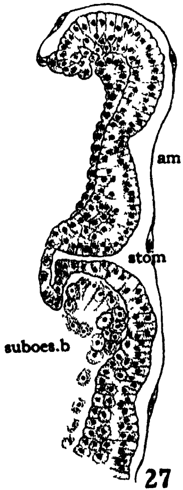

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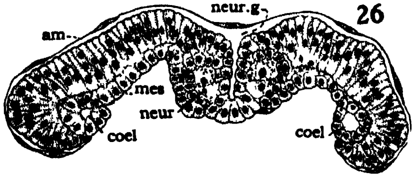

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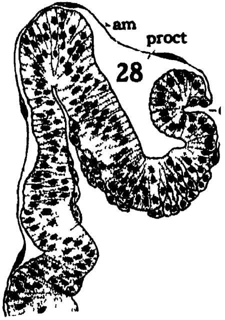

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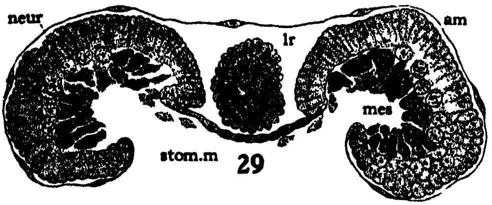

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PLATE 5

EXPLANATION OF FIGURES

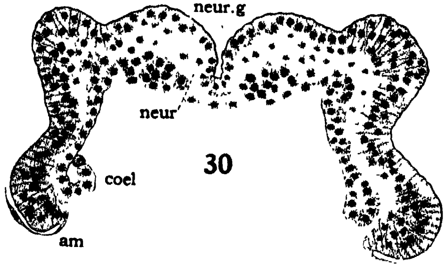
30 Cross-section through head between mandibles and maxillae of sixty-hour-old embryo. Amnion removed except on the margins. × 575.

31 Longitudinal section through proctodaeum of sixty five-hour-old embryo. × 525.

32 Cross-section through maxillary segment of head of sixty-hour embryo. × 600.

33 Longitudinal section of stomodaeum of sixty-five-hour embryo. × 600.

34 Oblique longitudinal section of proctodaeum of sixty-five-hour embryo. × 400.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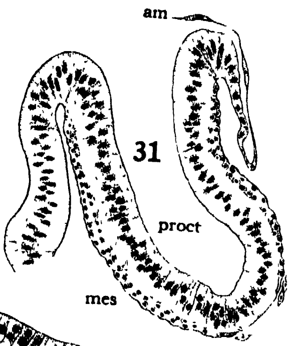
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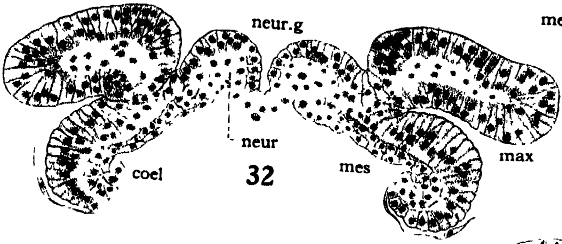
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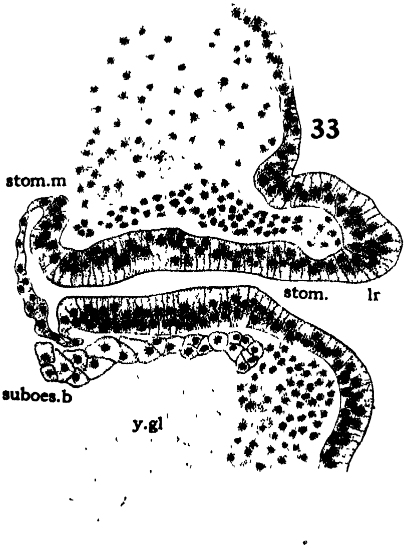
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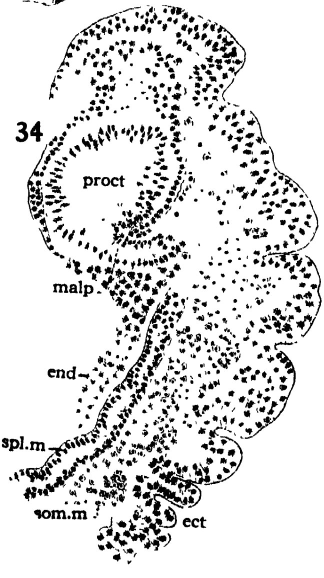
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PLATE 6

EXPLANATION OF FIGURES

35 Diagram of preprimordia of the germ layers, amnion, and serosa. The dark spots on the mesal margin of the ectoderm indicate neuroblast centers.

36 Cross-section of same when gastrula groove begins to form. Ventral side uppermost.

37 to 39 Successive stages in the formation of the gastrula groove, amnion, serosa. Ventral side uppermost.

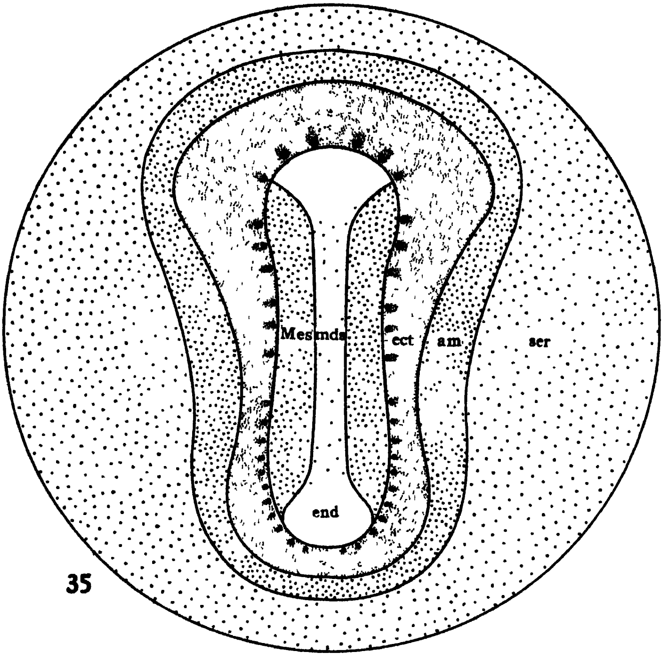
40 Formation of neural groove and liberation of the cells (*f.end*) of the middle strand and of the blood cells.

41 Longitudinal section corresponding to a stage a little later than in figure 39.

42 Formation of enteron rudiment when a complete gastrula tube is formed. Stomodaeal invagination beginning to form.

43 Formation of enteron rudiment when its cells are retarded in development until after the formation of a single-layered lower layer.

44 Formation of enteron rudiment when its cells are not retarded in development. Lower layer formed by overgrowth as shown in figure 43.



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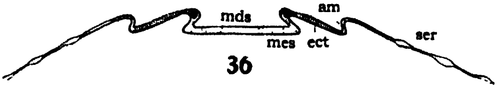

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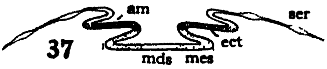

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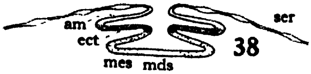

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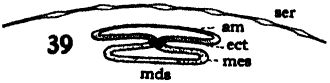

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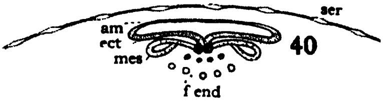

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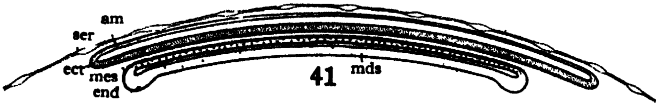

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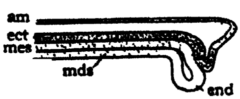

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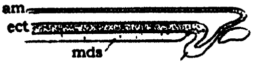

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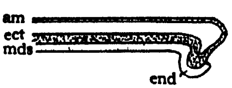

PLATE 7

EXPLANATION OF FIGURES

In figures 45 to 48 the ventral surface is directed upward and the amnion is removed, except on the margins. Embryos sixty-five hours old.

45 Section through the labial segment of the head. Apical portion of labium not shown. × 400.

46 Section through neck region posterior to the labium. × 500.

47 Section of mesothorax through the region of the spiracles. × 500.

48 Section through the mandibular segment. Only the base of the mandible is shown. Amniotic folds nearly meeting on the dorsal side. × 500.

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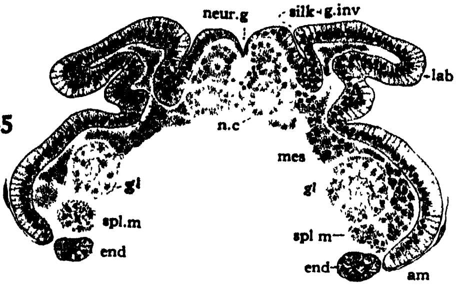
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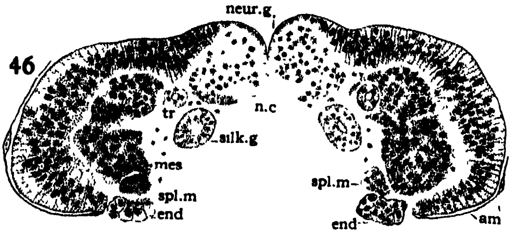
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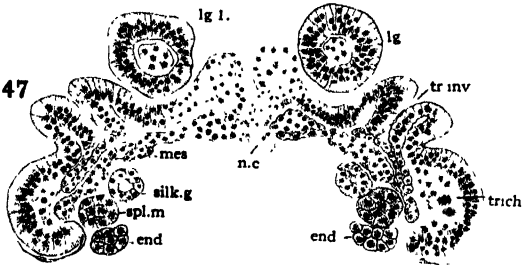
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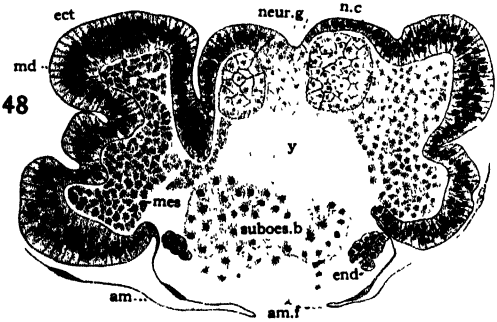
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PLATE 8

EXPLANATION OF FIGURES

49 Longitudinal section of stomodaeum cut somewhat tangentially, showing continuity of ectoderm and endoderm. Embryo seventy-seven hours old. × 500.

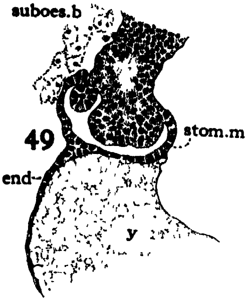
50 Longitudinal section of embryo. Owing to its slightly spiral position, the caudal end is obliquely cut. Amniotic fold (*am.f*) nearly meeting at the umbilical passage. First pair of prolegs (*p.lg*). Embryo eighty-two hours old. × 250.

51 Longitudinal section of suboesophageal ganglion and first thoracic ganglion (on the left). Ventral surface uppermost. Embryo eighty-two hours old. × 450.

52 Cross-section through posterior part of abdomen. Amniotic fold completely covering dorsal side. Amnion proper removed. Embryo eighty-two hours old. × 650.

53 Cross-section of abdomen in the region of the gonads, anterior to the proctodaeum. Neither the gut nor the body wall closed dorsally. Embryo eighty-four hours old. × 425.

54 Longitudinal section of stomodaeum, showing continuity of ectoderm and endoderm. Embryo eighty-two hours old. × 350.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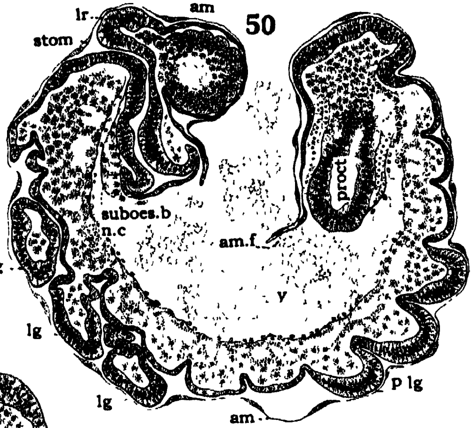
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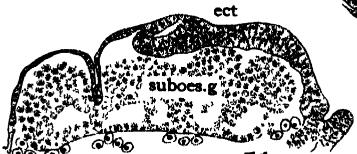
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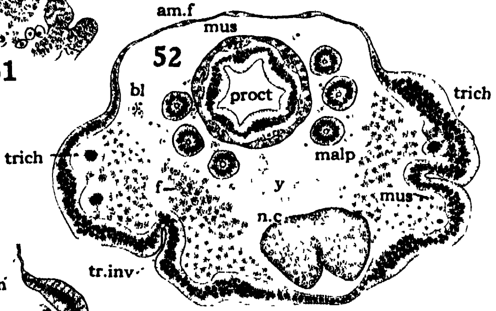
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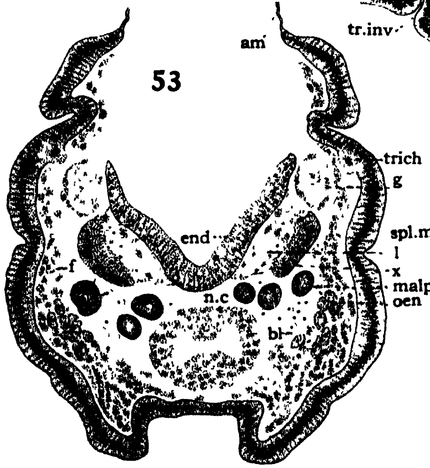
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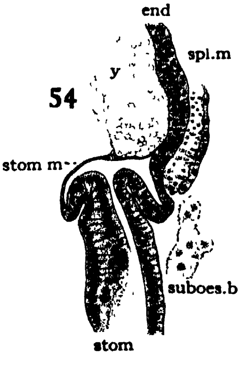
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THE PARIETAL FOSSA AND RELATED STRUCTURES IN THE PLAGIOSTOME FISHES

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Zoological Laboratory, Grinnell College

ELEVEN FIGURES

AUTHOR'S ABSTRACT

The parietal fossa or pit is situated in the middorsal line between the ear capsules. From its floor four (sometimes only two) apertures lead into the ear capsules. The anterior apertures are the foramina of the endolymphatic ducts; the posterior ones are the fenestrae. Through the fossa each endolymphatic duct passes from its foramen to its external aperture in the dorsal integument, describing in its course a loop with the convexity directed anteriorly. The part of the duct involving the loop is enlarged into an endolymphatic pouch. Into the angle of this loop a small muscle is inserted which is a continuation of the anterior trunk muscles, or one taking a more lateral origin from the edge of the fossa. The fenestra may or may not be closed by a definite fenestral membrane. The posterior semicircular canal (posterior utriculus) bears a peculiar relation to the fenestra. The endolymphatic pouch, like the sacculus, contains otoconia, and sometimes siliceous sand grains. Various functions have been assigned to the endolymphatic organ. There is no convincing experimental evidence as to its significance.

In 1927, Smiley, Ingram, and Blagg, students working under my direction, published a preliminary paper entitled, "The organs of the parietal fossa in elasmobranch fishes," a study based upon serial sections through the fossa in examples of six genera: Galeorhinus, Notorynchus, Rhina, Rhinobatus, Squalus, and Urobatis. Wax models of the endolymphatic duct and pouch and related muscle were constructed. The facts as demonstrated led to quite different conclusions from those of Davidson ('18) and Daniel ('22). The extension of the investigation to include ten more genera of elasmobranchs made it advisable to publish another account to include additional facts (Norris, '28). The present paper embodies an attempt to state the salient facts and to relate them to the literature of the last three centuries dealing with the structures in what we now term the parietal fossa of the plagiostome fishes.

To Prof. J. Frank Daniel, of the University of California, I am indebted for the Heptanchus (Notorynchus) material. The other material was collected by myself while enjoying the hospitality and privileges of the Scripps Biological Station at LaJolla, California, 1920–1921.

In the words of Daniel ('22):

> in the mid-dorsal line between the auditory capsules is the parietal fossa or pit (sharks), from the bottom of which certain apertures lead to the ear. Two of these are for the endolymphatic ducts and two other accessory apertures are the fenestrae for the perilymphatic spaces (fig. 1, *pf.;* fig. 2, *fed.* and *fstr.*).

The above is in complete agreement with the statements of Gegenbaur ('72), who seems to be the first to characterize definitely this depression ('Parietalgrube'). Davidson ('18) and Daniel both mention and figure a pair of small muscles in the parietal fossa of Heptanchus, attached, as they believe, to a membrane stretched across the fossa. The fossa and related structures will thus include the two pairs of openings in the floor of the fossa, the endolymphatic ducts with their external apertures in the skin overlying the fossa, the pair of small muscles mentioned above, and the possible membrane

ABBREVIATIONS FOR ALL FIGURES

aur., ear capsule
cspt., supratemporal lateral-line canal
ed., endolymphatic duct
eda., external aperture of endolymphatic duct
ede., external passage of endolymphatic duct
edi., internal passage of endolymphatic duct
ep., endolymphatic pouch
dpc., duct of posterior utriculus connecting with sacculus
du., ductus utriculi
fed., foramen of endolymphatic duct
fstr., fenestra
fstrm., fenestral membrane
hc., horizontal semicircular canal
int., integument
lvhmd., levator hyomandibulae muscle
ls., perilymphatic spaces bounded by definite walls
mdobl., medulla oblongata
pc., posterior semicircular canal = posterior utriculus of text
pf., parietal fossa
pm., muscle of the parietal fossa
rec.-utr., recessus utriculi
sac., sacculus
spr., spiracle
thm., thymus
tm., trunk muscles
tpm., tendon of muscle of fossa
utr., utriculus
VII, facial nerve
VIII, auditory nerve
IX, glossopharyngeal nerve
X, vagus nerve

or membranes stretched over or through the fossa or closing the fenestrae. In many instances the supratemporal latero-sensory canal passes over the posterior part of the fossa (fig. 4, *cspt.*). Much of the fossa may be filled with a fibro-gelatinous material. The fossa may be relatively deep, as in most of the selachoid forms, or shallow, as in most of the rays, or scarcely show a depression, as in some sting rays.

In this paper the term 'endolymphatic pouch' is applied to that enlargement of the endolymphatic duct commonly called the endolymphatic sac. Following Portmann ('20), this is done to avoid any seeming homologizing of this structure in elasmobranchs with the endolymphatic sac of higher forms.

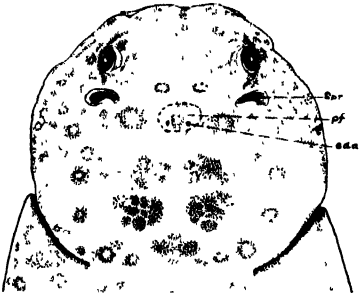

Fig. 1 Dorsal view of the head of an advanced embryo of Rhina californica. To show the position of the parietal fossa (*pf.*) and the external apertures (*eda.*) of the endolymphatic ducts. Natural size.

Scarpa (1789) seems to be the first to recognize the fossa as a feature of importance, although Geoffroy (1778) figures it indistinctly in Raia, and describes two apertures, an anterior smaller and a posterior larger, that evidently are the openings of the endolymphatic ducts and the fenestrae for the perilymphatic spaces (of Daniel). Camper (1774), Vicq d'Azir (1776), and Scarpa all denied that there is any communication between the ear and the exterior of the body in the cartilaginous fishes. Hence Scarpa overlooks the endolymphatic duct and its enlargement, or endolymphatic pouch, in the parietal fossa. He finds in both the flat (batoid) and cylindrical (selachoid) plagiostomes an oval fenestra, closed by a definite membrane, leading from the fossa on each side into the vestibule of the ear. This membrane, he says, lies just beneath the skin, here thinner than elsewhere, and serves as an aid in the propagation of sound. He mentions and figures in Raia a groove on the inner wall of the vestibule of the ear, at the top of which is the fenestra, a groove called tympanum by Howes ('83) and fenestral furrow by Daniel. Scarpa and also Weber (1820) show in their figures the characteristic relation of the posterior utriculus to this groove and to the fenestra in the skates. I have found, especially in the selachoid forms, that the posterior utriculus often fills or nearly fills the groove, and the wall of the posterior utriculus closes the fenestra wholly or in part. In the skates the posterior utriculus runs along the anterior border of the groove, and curves posteriorly around the anterior and dorsal borders of the fenestra.

That the membranous labyrinth in the plagiostomes communicates directly with the exterior seems to have been discovered independently by a number of observers. From some of these accounts it is difficult to conclude just what was seen. From Rondelet (1553), who says that fishes that have ears have only an auditory meatus (he doubtless refers to whales, not fishes); Casserius (1601), who evidently mistakes the olfactory nerve passage for an auditory meatus in the pike; Hans Sloane (1707), who describes in certain sharks holes in the back part of the head; to Swammerdamm (1737), who saw no external apertures of the ear in fishes, although in the German translation (1752) of his most noted work, Biblia Naturae, the word 'Eingang' is used where 'Irregang' should have been employed, we search in vain through the writings of the anatomists of these two centuries for any clear account of the ear of the plagiostomes, until we come to Monro (1785). He gave the first and adequate description of the ear of the cartilaginous fishes in its relation to the parietal fossa. Of the external apertures of the ear of the skate he says: "each of these leads into a capacious winding canal or concha which describes nearly a complete circle." He traces each 'concha' (endolymphatic pouch) into a cylindrical canal (endolymphatic duct) that passes into a large sac (sacculus). He investigated not only the ear of the skate, Raia, but also that of a selachoid form, Squatina (Rhina), finding the conditions in the latter essentially the same as in the skate. But of the relationships of the membranous vestibule to the parietal fossa and to the exterior in the selachoid plagiostomes in general Monro was very much in doubt. Both his text and illustrative plates are remarkably clear and exact, and it seems strange that his findings should have been rejected by all his contemporaries, except John Hunter (1782). The latter had believed that he himself was the discoverer of the external part of the plagiostome ear, but later came to believe that Willughby, in the seventeenth century, had been the first to see the external auditory pores in the skate, although he did not at all understand their significance.

The uncertainty whether the ear of the cylindrical plagiostomes opens to the exterior, as it does in the skate and other batoid forms, was strangely persistent. Cuvier (1799), Cuvier and Valenciennes (1828), Weber (1820), and Baly's translation of Müller's Physiology ('48), all restrict the external auditory pores to the flat plagiostomes. Weber's most important contribution to our knowledge of the structures in the parietal fossa is the discovery of the small muscle inserted on the endolymphatic pouch. The most distinctive feature of Breschet's ('38) work was his insistence on the integrity of the endolymphatic duct from sacculus to external aperture in the dorsal integument. He believed that conditions in the selachoid forms were essentially the same as in the skate. Leydig ('52) declares that Weber's description of the ear in the ray applies to all plagiostomes. To Hasse ('73 a and b) we are indebted for the terms 'ductus endolymphaticus' and 'saccus endolymphaticus.' He recognized the external aperture of the ear in both the selachoid and the batoid types, but strangely enough believed that the ductus endolymphaticus rising from the sacculus ends blindly in a sac-like swelling, filled with calcareous material, beneath the integument, although he was never able to find this supposed blind sac. The external aperture through the integument, he believed, leads into a space whose lining, derived from the wall of the vestibule and everted by the outgrowth of the endolymphatic duct (blind swollen end), extends over and envelopes the sac-like ending of the duct. Hasse also believed that the endolymphatic duct arises in part from the utriculus as well as from the sacculus, in this respect supporting the views of Ibsen ('46) and Hellmann ('98). Retzius ('78, '81) established once and for all time that the ductus endolymphaticus of all the plagiostomes ascending through its foramen in the cranial wall (floor of parietal fossa) never ends blindly, but opens freely to the exterior through the dorsal integument. Thus was ended the controversy which for nearly a century had retarded recognition of the fundamental character of the endolymphatic duct. Thus was reestablished the truth of Monro's long-neglected discoveries. Retzius also showed that the muscle of the parietal fossa occurs in both sharks and rays, completely corroborating Breschet's suggestions. In brief, he established the complete homology that exists between the ears of the selachoid and batoid plagiostomes. His belief that the ductus endolymphaticus arises solely from the sacculus in the plagiostomes requires some qualification. From a study of serial sections through the ear capsule and contents I find that the enlarged conical base of the ductus endolymphaticus, or recessus vestibuli, opens freely into the sacculus and recessus utriculi in the selachoid forms and in the true skates. In the sting rays, however, in which the recessus utriculi communicates with the utriculus by a very narrow duct, but opens widely into the sacculus, the endolymphatic duct connects with the sacculus only. This is very clearly shown by Retzius in his figure of the ear of the sting ray, Trygon (Dasybatus) pastinaca.

Owen ('66) reproduces Breschet's figure of the ear of Raia, showing endolymphatic duct and pouch and related muscle. Wiedersheim ('83) refers to the endolymphatic duct of selachians as under the control of a small muscle. Luther

('09) shows incidentally this muscle in Rhina. In general, recent text-books in comparative anatomy omit all reference to this small muscle. And so we find it rediscovered by Davidson ('18) and Daniel ('22).

In the parietal fossa and related structures of Squalus acanthias we see a simple but characteristic arrangement of parts (fig. 2): two pairs of openings in the floor, an anterior smaller pair (*fed.*) through which pass the endolymphatic ducts, and a posterior larger pair (*fstr.*), the fenestrae, whose openings are closed by the walls of the posterior utriculus (fig. 3). As Leydig observed, the endolymphatic duct does not go directly from the outer aperture in the skin to the inner opening (foramen of the endolymphatic duct) in the skull, but makes a loop with the convexity directed forward. In the angle of the loop is inserted the muscle of the parietal

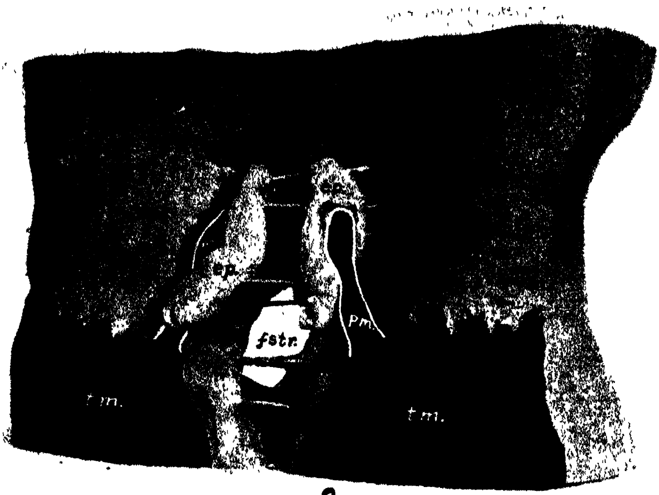

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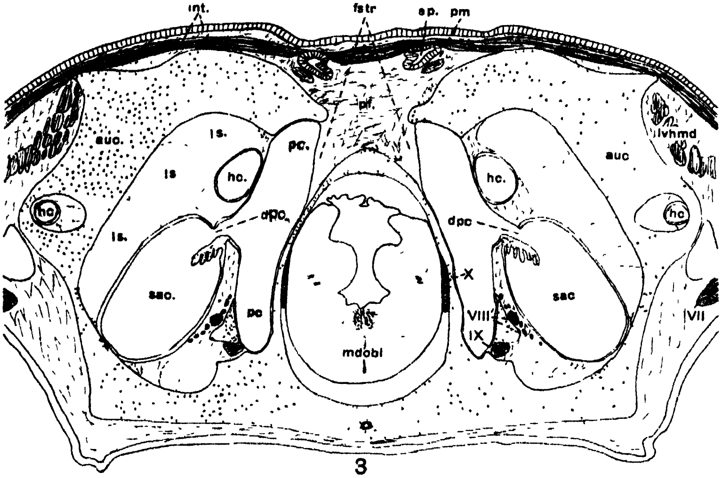
Fig. 2 Obliquely dorsal view of the parietal fossa and associated organs of an embryo of Squalus acanthias, 'pup' stage. From a photograph of a wax model by Elizabeth Blagg. The oblique view was taken in order to show a fenestra (*fstr.*). The muscle of the fossa (*pm.*) have been outlined in white. The integument has been omitted. × 13.3.

fossa (fig. 2, *pm.*), the latter being a continuation of the anterior trunk muscles (*tm.*). The cavity of the relatively deep fossa is occupied by a fibrogelatinous material in which the structures mentioned above are embedded. We may thus designate a number of parts in what may be called the endolymphatic organ: external aperture (*eda.*); external passage (*ede.*); the loop or endolymphatic pouch (*ep.*); the internal passage (*edi.*); the muscle (*pm.*). The loop or pouch is not sharply distinct from the rest of the endolymphatic canal in Squalus, but in Carcharinus (fig. 5) and generally in the rays (fig. 6) it is a conspicuous enlargement. In relation to each other the two limbs of the loop may lie in a nearly vertical plane (figs. 2 and 7) or in a nearly horizontal plane (figs. 5 and 8). The muscle of the fossa originates typically from the anterior trunk muscles (fig. 2), but in the rays and other flat plagiostomes (fig. 6) and in some cylindrical forms (fig. 5) from the lateral border of the fossa. The insertion is variable: typically in the angle of the loop, but in some instances on one or both limbs of the loop.

Howes' (83) statement that in Raia the fenestra enters a well-defined cavity or tympanum in the ear capsule is hardly of general application. In Raia and all other genera of plagiostomes examined by me, wherever the fenestra occurs at all the wall of the posterior utriculus closes the internal orifice of the fenestra or is situated but a short distance away internally. In most of the forms examined the wall of the posterior utriculus effectively closes the fenestra. The posterior utriculus lies in a vertical groove hollowed out of the inner cartilaginous wall of the ear capsule. In Raia, however, this anterior part of the posterior utriculus does not lie in the groove, but curves around its anterior border. This groove was figured by Scarpa and by Weber in Raia and its relation to the posterior utriculus correctly shown, and is beyond question the so-called tympanum of Howes and the fenestral furrow of Daniel.

As to the presence in the parietal fossa of plagiostomes of a structure analogous in function to a tympanic membrane,



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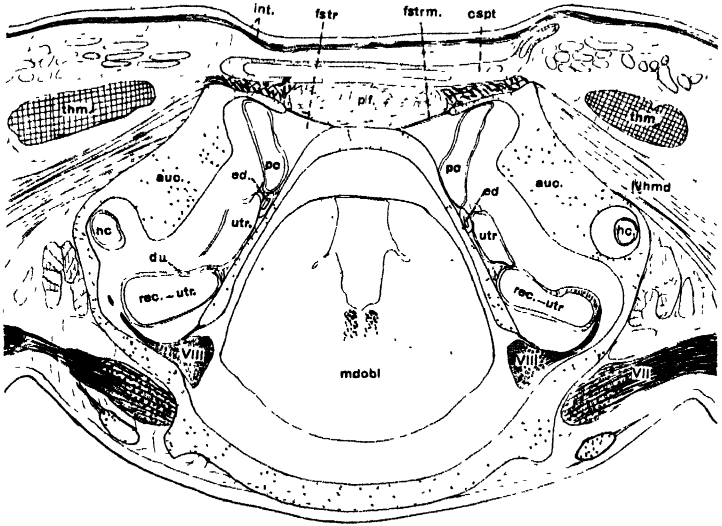

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Fig. 3 Cross-section through the ear capsules and parietal fossa of Squalus acanthias, 'pup' stage. Shows the characteristic relation of the posterior utriculus (*pc.*) to the fenestra in the selachoid plagiostomes. × 10.

Fig. 4 Cross-section through the ear capsules and parietal fossa of Urobatis halleri, advanced embryo. Shows the fenestra closed by a fenestral membrane (*fstrm.*), and also in part by the wall of the posterior utriculus (*pc.*). The characteristic relation of the supratemporal laterosensory canal (*cspt.*) to the fossa is seen. × 10.

as Daniel believes, there is room for differences of opinion. But the facts compel one to deny that there is a membrane stretched across, just beneath the skin, its tension regulated by a pair of small muscles, as Daniel concludes for Heptanchus. This pair of small muscles is invariably inserted on the endolymphatic pouches. It is true that a membrane usually closes the fenestra (fenestra ovalis of Scarpa) in the floor of the fossa, but a definite fenestral membrane, forming a part of the wall of the fossa, does not always exist. In some instances, as in Cephaloscyllium, Eugaleus, Notorynchus, Rhina, and Squalus (figs. 3 and 11), the membranous wall of the posterior utriculus, as stated above, closes the fenestra more or less completely, and not a distinct fenestral membrane. In Galeorhinus and Urobatis there is in addition to the posterior utriculus wall a true fenestral membrane (fig. 4). A fenestral membrane is common in the ray-like forms. The fenestra is lacking in Centracion, Pteroplatea, and Syr-

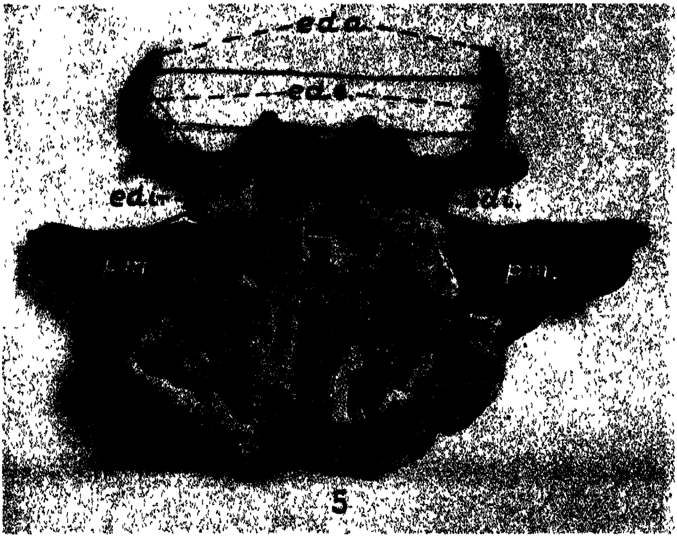
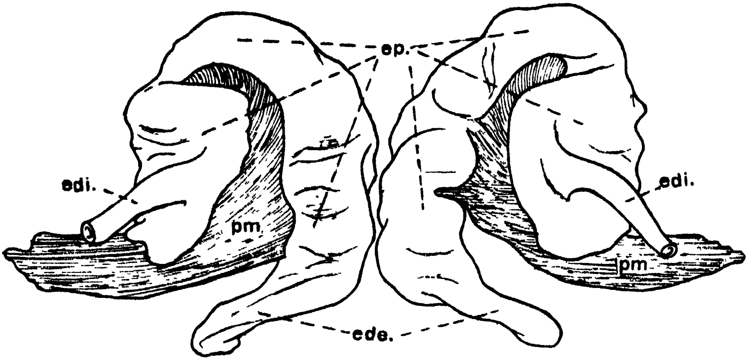


Fig. 5 Ventral view of the endolymphatic organ of Carcharinus commersonii, 'pup' stage. From a photograph of a wax model by Orville Sharnborg. × 7.5.

rhina, probably united with the foramen of the endolymphatic duct. In Myliobatis the fenestra is completely covered by the anterior extension of the trunk muscles. In the rays the fossa is shallow, in Myliobatis and Pteroplatea practically



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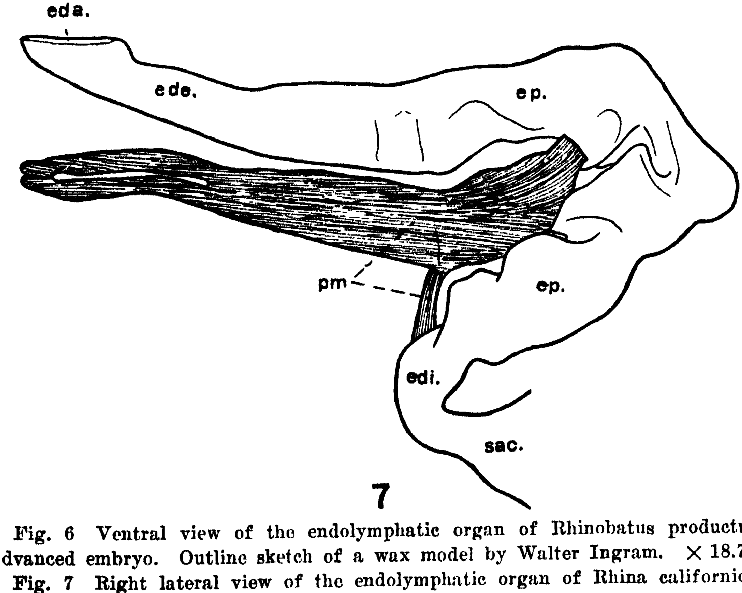

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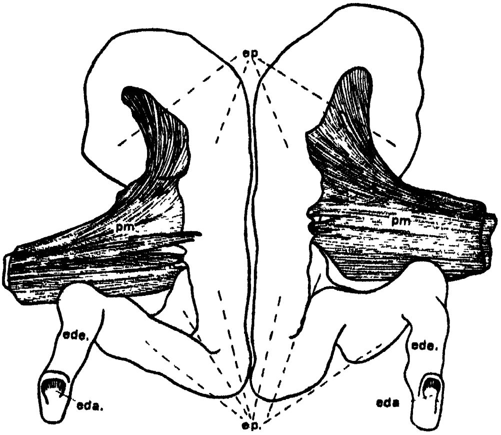
Fig. 6 Ventral view of the endolymphatic organ of Rhinobatus productus, advanced embryo. Outline sketch of a wax model by Walter Ingram. × 18.75.

Fig. 7 Right lateral view of the endolymphatic organ of Rhina californica, advanced embryo, same stage as shown in figure 1. Outline sketch of a wax model by Ralph Smiley. × 28.5.

non-existent, the endolymphatic pouch and its muscle flattened out between the skin and the skull.

I have found the endolymphatic duct and its opening to the exterior and the endolymphatic pouch with its attached muscle present in the following genera: Carcharinus, Centracion (Heterodontus), Cephaloscyllium (Catulus), Eugaleus,



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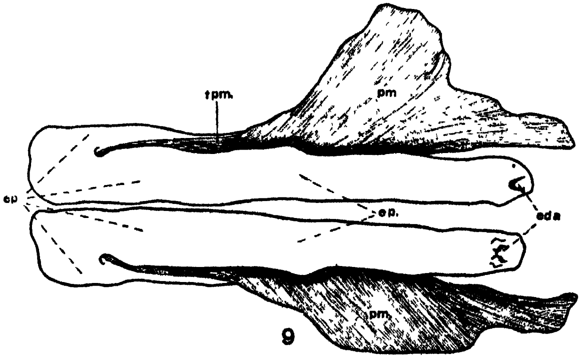

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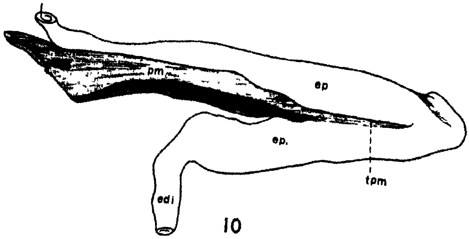
Fig. 8 Dorsal view of the endolymphatic organ of Galeorhinus lunulatus, 'pup' stage. Outline sketch of a wax model by Walter Ingram. × 15.

Fig. 9 Dorsal view of the endolymphatic organ of Notorynchus platycephalus, adult. Outline sketch of a wax model by Elizabeth Blagg. × 5.

Galeorhinus (Mustelus), Notorynchus (Heptanchus), Rhina, Squalus, Triakis, Myliobatis, Platyrhinoidis, Pteroplatea, Raia, Rhinobatus, Syrrhina (Zapteryx); and Urobatis (Urolophus). In no species of the plagiostomes examined



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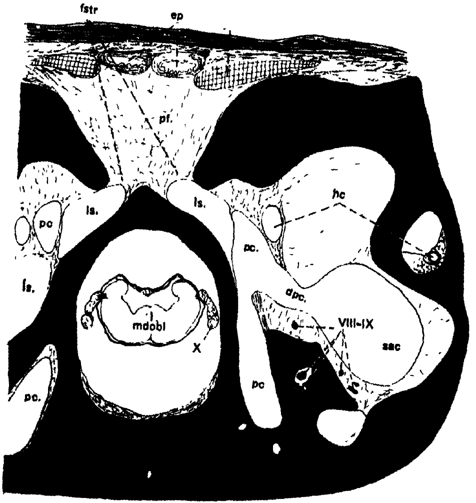

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Fig. 10 Right lateral view of the preceding. × 5.

Fig. 11 Cross-section through the parietal fossa and left ear capsule of Notorynchus platycephalus, adult. (All cartilage is represented in black.) The wall of the posterior utriculus partly closes the fenestra. No definite fenestral membrane. There is a close resemblance to the condition in Squalus (fig. 3), but the wall of the posterior utriculus does not so completely close the fenestra as in Squalus. Lymph spaces (perilymphatic passages) are more sharply defined, passing posteroventrally from the fossa, through the fenestra, into the vicinity of the posterior utriculus. × 3.

by me have they been absent, or even rudimentary. We may therefore consider them present in, and characteristic of all, the plagiostome fishes. Stewart ('06 b) reports that he found no external auditory pores in Centracion philippi, but Daniel finds them in Centracion francisci.

Some of the early accounts of the ear of fishes deal largely with the earstones or otoliths found in the vestibule of the ear. As early as Willughby, in the seventeenth century, according to the testimony of Weber, these bodies in the ear of cartilaginous fishes were recognized as being of a different structure from corresponding bodies in the ear of bony fishes. Instead of hard pebble-like otoliths, they take the form of soft chalky masses cohering through amylaceous material ('mucus-connected, calcareous particles,' Bridge, '22). Breschet designated them 'otoconia,' and as such we know them to-day. Monro, in his discovery of an external opening of the ear of the ray, appears to be the first to recognize the presence of similar chalky material in the endolymphatic pouch. Daniel states that in the parietal fossa of preserved specimens of Heptanchus occur many granules, coagulations of the original fluid contents of the fossa. These I find are confined to the endolymphatic pouch and duct and the latter's continuation into the sacculus, and do not occur in the fossa itself. Daniel notes also that sand grains are reported to be found in the sacculus of adult Squatina. Stewart ('06 b) says that in Rhina (Squatina) the otoconia of the sacculus are replaced by sand grains introduced through the endolymphatic duct. He also states that in Acanthias (Squalus) vulgaris a mixture of otoconia and sand grains occurs. Weber reported that in Torpedo marmorata black sand was mixed with the gelatinous mass in the endolymphatic pouch. I have found siliceous as well as calcareous grains filling the endolymphatic pouch of adult Squalus acanthias, these grains extending down into the sacculus. So abundant is the siliceous sand in adult Squalus acanthias that I have found it well-nigh impossible to cut sections of the pouch. In advanced embryos of Rhina californica, taken from the oviduct of the parent, I have invariably found siliceous sand grains in the endolymphatic pouch extending down into the sacculus. This seemingly improbable condition is not so strange when we consider the free communication of the oviduct with the cloaca in this form and the possibility of free movements of the embryos in the oviducts, for in Rhina the oviducts are little more than brood sacs. I have found siliceous sand grains in the endolymphatic pouch of adult Centracion, Squalus, and Platyrhinoidis and in embryos of Rhina. I have failed to find them in adult Heptanchus, Myliobatis, Raia (two species), and Syrrhina. Adults of other genera I have not examined for these bodies. I find remains of otoconia in the endolymphatic pouch of most embryos and all adults examined.

Scarpa regarded the fenestra of the parietal fossa as equivalent to the fenestra ovalis of the ear of the air-breathing vertebrates. Weber considered it a fenestra rotunda, and that, without doubt, sound waves were propagated through the skin overlying the fossa into the cellular web filling the cavity and by the membrane stretched over the fenestral opening transmitted to the fluids surrounding and filling the membranous vestibule. He considered the foramen through which the endolymphatic duct passes as the equivalent of a fenestra ovalis. He believed that the endolymphatic pouch, by its connections with the membranous labyrinth through the foramen, could function like the auditory ossicles of the air-breathing forms. A number of writers, notably Gegenbaur ('72) and Garman ('13), have in their delineations of the plagiostome skull shown more or less incidentally the parietal fossa and the openings in its floor. Little attention was paid by these writers to the openings, and they are usually portrayed more or less casually, so that in some instances it is impossible to determine whether any attempt was made to represent fenestral apertures. Existing apertures are not always shown, but when four such are indicated, that may be taken as the correct number. Taking these various lists of plagiostome cranial illustrations, relating them to my own observations, eliminating duplicates, and clearing up the synonomy, I have a list of thirty-nine genera, in twenty-eight of which the number of openings in the floor of the fossa is four. Of the remaining eleven there is uncertainty about six of them, with the probability that most of the six have four apertures. Five of the thirty-nine genera appear to have the fenestra combined with the foramen of the endolymphatic duct.

Portmann ('20), basing his investigations on Torpedo and Myliobatis, describes the wall of the endolymphatic organ as smooth and compact at the level of the external passage, becoming extremely folded in the endolymphatic pouch, taking at some points the aspect of villosities on the inner surface, again becoming smooth and compact in the duct proper and the sacculus. This description by Portmann is very characteristic of the structure of the endolymphatic organ in the batoid plagiostomes. I am unable to agree with Portmann in his description of the epithelium lining the duct and pouch. In all the species examined by me, including Myliobatis, I find the epithelium of the endolymphatic organ is a single layer of columnar cells, in contrast to a many-layered condition, as described by Portmann, in some parts of the organ. Between the endolymphatic pouch and the sacculus the epithelium gradually flattens down to a squamous type. My own observations here are in complete agreement with those of Retzius. The columnar epithelial cells of the endolymphatic organ appear to have taken the form of rod-like prisms that give a rigid form and constant shape to the organ, keeping the canal open and unobstructed. Breschet refers to the fibrocartilage resistance of the wall of the external passage.

Varying and conflicting explanations have been given of the supposed functions of the structures in the parietal fossa. No attempted explanation that ignores the presence of the muscles of the fossa can be seriously entertained. The only experimental evidence so far presented is that of Portmann ('21), who is apparently wholly unaware of the existence of these muscles. On closing the external aperture of the endolymphatic duct by injection of melted paraffin or by electric cautery, he found that the animals (Dasybatus pastinacus) lost all power of orientation and equilibrium. In the muscles of the parietal fossa Weber saw the mechanism for the expulsion of the contents of the pouch. Breschet suggested that these muscles may serve to increase the tension of the overlying skin and thus facilitate sound propagation. Daniel, as we have seen, has much the same opinion. In the opinion of Hellmann ('98), the chief use of the endolymphatic duct is to secure the drainage of superfluous labyrinth material, and its chief biological interest lies in its development. We have yet to explain the use of sand grains in duct, pouch, and sacculus. The muscles of the fossa must be an active agency in this intake of foreign material. But it must be shown that this appropriation of sand grains is of more general occurrence than facts at present indicate. It has long been the opinion that hearing is not the chief function of the ear in fishes. The ear is but a part of a more widely distributed system of organs, the acusticolateral system, whose fundamental reactions seem related to the perception of molar vibrations of matter. Sensations of pressure, equilibrium, and audition all seem related to this system of sense organs. That the endolymphatic duct and its attached muscle may have some relation to the adjustment of the internal ear to varying pressures of water seems not improbable. But I see no reason for contesting the views of Daniel and of Howes that a fenestral membrane may promote sound perception in the cartilaginous fishes.

It is to be hoped that some experimenter, skilled in the technique necessary for the investigation of the equilibratory functions of the acusticolateral organs, will turn his attention and research toward the solution of the problem of the function of the endolymphatic organ in plagiostomes, for it is evident that the endolymphatic duct is a structure of wider interest than merely as a persistence of an early embryonic condition.

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ON THE STATOCYST OF THE AMERICAN CEPHALOPOD GENERA

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ONE DIAGRAM AND TWELVE FIGURES

AUTHOR'S ABSTRACT

From the structure of statocysts, in the so-called phylogenetic tree of the Cephalopoda, the genus Scaeurgus is to be placed on the same branch as Polypus

Meleagroteuthis has the same number of processes as Watasenia, Enoploteuthis, Abralia, Illex, and Stigmatoteuthis, which have ten processes, but in this genus the structure of statocysts seems to be tolerably well developed For this reason, the author places this genus as a branch between Abralia and Illex

Those three genera—Gonatus, Sthenoteuthis, and Onychoteuthis—which have eleven processes in a statocyst are to be placed in the same branch as Berryteuthis, Gonatopsis, Ommastrephes, Eucleoteuthis, Moroteuthis, and Symplectoteuthis, which have the same number of processes, of these, Gonatus, which has the most primitive structure of the statocyst, is to be put in the lowest position on that branch, but Sthenoteuthis, which has the same structure of the organ as Ommastrephes, is to be placed on the twig near Ommastrephes, and, lastly, Onychoteuthis has the same organ as that of Moroteuthis, which fact induces the author to set this genus in the position nearest to Moroteuthis.

The statocyst of Lolliguncula corresponds exactly to that of Loligo, which has twelve processes, this condition puts this genus on the same twig as Loligo.

In a recent work[1] published by the writer, the comparative morphology and the phylogenetic meaning of the structure of statocysts in different genera and species of the cephalopods of Japan were discussed. In the present paper the writer presents the results of his studies of certain American cephalopods from the Pacific and other oceans.

These include thirteen genera and twenty-two species and add six genera to the so-called phylogenetic tree previously derived from a comparative study.

This work was done at Leland Stanford University under a grant from the International Education Board. The writer wishes to express his appreciation of the courtesies extended to him by the Stanford University Museum, and especially by Dr. David Starr Jordan, Dr. John Otterbein Snyder, and Dr. Harold Heath.

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[1] 1924 The Journal of the College of Agriculture, Imperial University, Tokyo, Japan, vol. 7, no. 3, pp. 165–210; plates VIII–XIII.

METHOD OF OBSERVATION

As the preserved specimens were comparatively large, it was easy to cut open the base of the siphon, take out the statocysts, together with the head cartilage, to divide the cavity transversely at the position it assumes in the head of the animal, and to observe the statocysts from the inner side under a dissecting microscope. The figures in the text are all somewhat diagrammatic, and the reference letters have the same meaning throughout (figs. 1 to 12). For convenience, the author will also employ abbreviations.

GENERAL STRUCTURE OF THE STATOCYSTS

The structure of the statocysts of the Octopoda (fig. 2 or fig. 3) is very simple. In the region of the comparatively large elliptical macula statica (*ms*) the tissue is denser and thicker than in any other part of the epithelial cyst (*epc*); it may be seen distinctly under a dissecting microscope. The nerves coming from the cerebral ganglion may be observed running along the line corresponding to the long diameter of the macula statica. On the macula statica is mounted a conical statolith, which curves in a hook shape and has as broad an elliptical base as the macula statica (*ms*). Opposite the macula statica, near the median wall of the statocyst, there is one process which we designate as *c;* however, in case of the Octopoda, this process appears in many different forms, such as a pliant, simple finger, or a thin, flat, and pliant form, or globular, or a finger with many small warts on the whole surface, or with its terminal point bearing two or more irregular projections. The shape seems to vary more or less according to the individual or according to the growth of the animal; but the place on whch the process *c* grows is invariable in the case of the statocysts of the Decapoda. What the author calls the *c* process, located at the same position as the *c* process of the Octopoda, is simply a process shaped like an elongated, strongly curved, finger-like cone, which is not so pliant as that of the Octopoda. The crista statica (*cs* in fig. 2) which is the ridge of the epithelial cyst (*epc*) arises anteriorly in the vicinity of the macula statica, runs toward the side and, returning to the median wall, passes the outer and under side of the *c* process, and finally ends very near the macula statica, giving out some twigs on the way. On the whole, it makes an even spiral on the epithelial cysts. Further, there may be perceived the nerves coming to the crista statica from the cerebral ganglion and, in the perilymphatic sinus (*ps*), the meshes of connective tissues supporting the epithelial cyst, and the net-like blood vessels going into the meshes and extending in the epithelial cyst. In the Decapoda the epithelial cyst (*epc*) is closely adherent to its surrounding cartilage (*ca*), and the perilymphatic sinus (*ps*) between the epithelial cyst and the cartilage cannot be perceived as in the Octopoda. This is the general construction of the statocyst, which can be seen under a dissecting microscope.

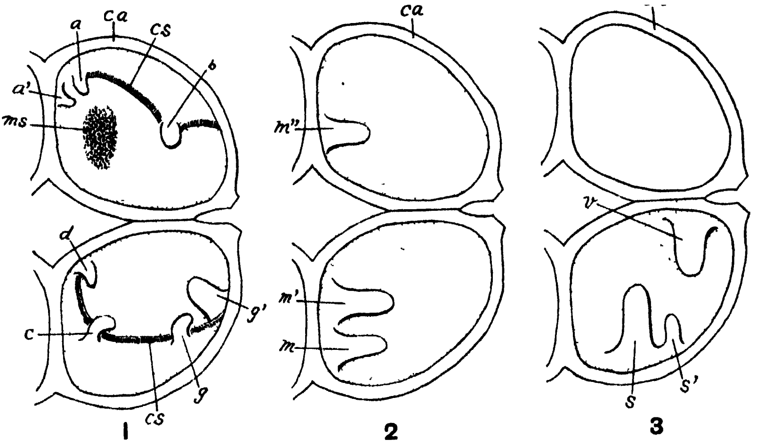

Fig. 1 Diagram of dissections of the left statocyst of Decapoda; enlarged. 1. Processes in the first region only are shown. 2. Processes in the second region only are shown. 3. Processes in the third and fourth regions. *a, a', b, c, d, g, g', m, m', m'', s, s'*, and *v*, processes; *ca*, cartilage; *cs*, crista statica; *ms*, macula statica.

The statocysts of the Decapoda (fig. 1) are more complicated in their structure than those of the Octopoda; the epithelial cyst is not a simple sphere, especially when the animal is fully grown; the inner side of the statocyst is irregularly shaped, since each process is enlarged, nearly filling and greatly narrowing the inner space of the statocyst. The macula statica (*ms*) does not form a true elliptical plane, and under a microscope of lower power cannot be seen as distinctly as the macula statica of the Octopoda; but it appears near the median wall of the head part when the statocysts are separated into two parts as in the Octopoda. The crista statica (*cs*) appears as a high ridge at about the same position as in the Octopoda and makes a curve on the inner side of the epithelial cyst; but, as shown in the figure (fig. 1, *1*), many processes are developed on both sides of the ridge in what we will call the first region. Seven processes occur in this region: *a, a′, b, c, d, g,* and *g′*, as shown in the figure (fig. 1, *1*).

The processes of the second region grow on both sides of the median wall of the statocyst, and are separated from those along the crista-statica line. They are three in number, designated *m, m′*, and *m″* in the figure (fig. 1, *2″*). The processes of the third region grow on the wall of the siphonal side of statocysts, and they are generally two in number, *s* and *s′*, as shown in the figure (fig. 1, *3*). In the fourth region is one process, *v,* which is located on the wall of the statocyst, placed in close contact with the visceral ganglion (fig. 1, *4*).

In general, each process of the first region is distinctly smaller in size than those of other regions, and two processes, *c* and *g,* differ from others in being hooked so as to cover completely the sense cell on the crista statica; the processes of the second region are comparatively large, all about the same size, and straight; the processes of the third region are two, one large and the other small; the fourth region has only one process, which is comparatively blunt and short. In the following pages the structure of the statocyst in each species is described in greater detail.

OCTOPODA

Of the Octopoda, the author examined the structure of the statocysts of seven species belonging to Polypus and of one species belonging to Scaeurgus.

Polypus bimaculatus (*Verrill*) (fig. 2)

This species, common along the coast of southern California, bears remarkable ocellated spots near the base between the second and third arms, just in front of and below the eye on either side. In a small specimen of a total length of 70 mm. and a mantle length of 17 mm., the perilymphatic sinus (*ps*) between the epithelial cyst (*epc*) in the statocyst and the surrounding cartilage (*ca*) is extremely narrow; but in a specimen with a length of 200 mm. and a mantle length of 30 mm. the perilymphatic sinus is enlarged, and the longest diameter of the epithelial cyst is about one-half of the longest diameter of the perilymphatic sinus. The development of the perilymphatic sinus when the animal approaches maturity is faster than that of the epithelial cyst, as in other species of Polypus as well as in Argonauta—a fact which the author

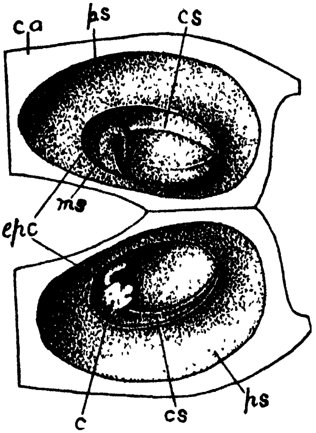

Fig. 2 Polypus bimaculatus, dissections of statocyst of a specimen with a mantle length of 30 mm. (sp. no. 2). × 8. The lettering is explained in the text.

has already indicated in the statocysts of Japanese specimens. The statolith is a hook-like, curved cone, and the base is mounted on the macula statica (*ms*) in an elliptical shape. Opposite the statolith, close by the median wall, there is a process, *c*, which is very flat, and is separated into three projections at its tip, the middle one being somewhat higher than the others. The crista statica (*cs*) extends toward the outside from under the process *c* and is divided into two parts, one reaching very near to the macula statica; these structures bear a close resemblance to those of Polypus areolatus.

Specimens examined

No.	*Locality*	*Sex*	*Collectors*	*Where deposited*
1	San Diego, California	Female	E. C. Starks	Stanford University
2	La Jolla, California	Female	San Diego Marine Biological Assn.	Stanford University

Polypus apollyon Berry

Specimens examined

No.	*Locality*	*Sex*	*Where deposited*
3	Pacific Grove, California	Female	Stanford University
4	Pacific Grove, California	Male	Stanford University

Polypus macropus Hoyle

Specimens examined

No.	*Locality*	*Sex*	*Collectors*	*Where deposited*
5	Hakodate, Japan	Female	Jordan and Snyder	Stanford University
6	Bay of Waka, Kii, Japan	Male	Jordan and Snyder	Stanford University

In these two species the structures of the statocysts are quite the same as those of Polypus bimaculatus, as described above; the process *c* is also thin and flat and has three small wart-like projections at its tip.

Polypus oliveri Berry

In this species the epithelial cyst of the statocyst is much smaller than that of other species belonging to this genus. In a specimen with a mantle length of 30 mm. and a total length of 165 mm., the diameter of the epithelial cyst measures only 1.5 mm., but otherwise the statocyst is exactly like those of the above-mentioned species.

Specimen examined

No.	*Locality*	*Sex*	*Collector*	*Where deposited*
7	Sunday Island, Kermadec Group	Female	W. R. B. Oliver	Stanford University

Polypus ornatus (*Gould*)

The structure of the statocysts in this species is exactly like that of Polypus bimaculatus; but the process *c* has such elongated projections that it seems to be three processes united into one at the root of each process.

Specimen examined

No.	*Locality*	*Sex*	*Collector*	*Where deposited*
8	Honolulu, Hawaii	Male	Albatross Expedn.	Stanford University

Polypus marmoratus Hoyle

In this species the structure of the statocyst is, in general, the same as that of Polypus bimaculatus, while the process *c* is thin and flat and has a few irregular warts at its tip.

Specimen examined

No.	*Locality*	*Sex*	*Collector*	*Where deposited*
9	Honolulu, Hawaii	Male	Jordan and Evermann	Stanford University

Polypus punctatus (*Gabb*)

In both the specimens with a mantle length of 210 mm. and of 240 mm., the process *c* of the statocyst has many warts along the whole body, while in a small animal with a mantle length of 50 mm., as stated already ('24, p. 172), it is a simple cone without any wart.

Specimens examined

No.	*Locality*	*Sex*	*Collector*	*Where deposited*
10	Point Lobos, Calif.	Male	M. Ishikawa	Stanford University
11	Point Lobos, Calif.	Male	M. Ishikawa	Stanford University

Scaeurgus patagiatus Berry (fig. 3)

In a specimen with a mantle length of 28 mm. and a total length of 140 mm., the structure of the statocyst is quite the same as in the genus Polypus. The process *c* of the statocyst is exactly similar to that of Polypus marmoratus, which is thin and flat and, at the tip, separated into irregular projections. This fact shows that the animal is to be placed in the vicinity of Polypus.

Specimen examined

No.	*Locality*	*Sex*	*Where deposited*
12	Hawaiian Island, Albatross Station 4103	Female	Stanford University

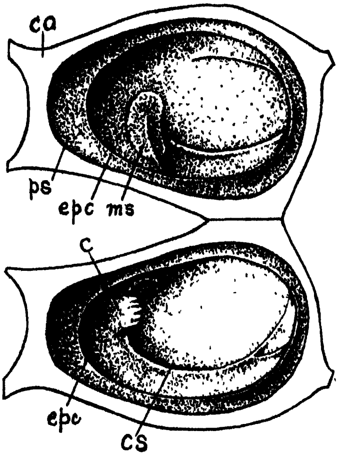

Fig. 3 Scaeurgus patagiatus, dissections of statocyst of a specimen with a mantle length of 28 mm. (sp. no. 12). × 11. The lettering is explained in the text.

DECAPODA

On the Decapoda the structure of the statocyst for fourteen species belonging to eleven genera observed by the author is as follows:

Euprymna scolopes Berry (fig. 4)

This is a very common species found in Hawaiian waters. The structure of the statocyst is similar to that of the common Japanese species Euprymna morsei. In a specimen with a mantle length of 19 mm. the number of processes is four in the first region, i.e., *a, c, d,* and *g,* of which *c* and *g* are strongly curved and slightly larger than *a* and *d*. In the second region only a larger process, *m*, is found; in the other regions none is perceived.

The structure of these statocysts must certainly be primitive, as already stated ('24, pp. 181 and 199), compared with that of other Decapoda.

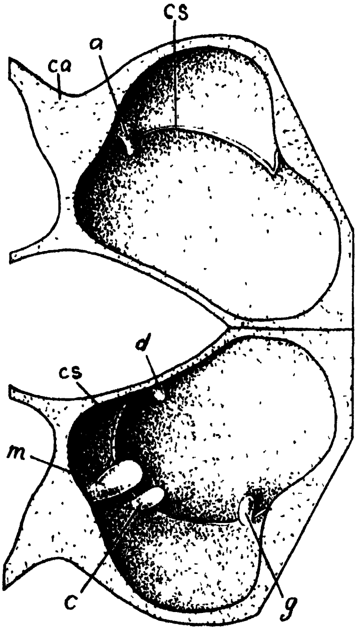

Fig. 4 Euprymna scolopes, dissections of statocyst of a specimen with a mantle length of 19 mm. (sp. no. 13). × 20. The lettering is explained in the text.

Specimens examined

No.	*Locality*	*Sex*	*Collector*	*Where deposited*
13	Honolulu Reef, Hawaiian Islands	Female	O. P. Jenkins	Stanford University
14	Honolulu, Hawaii	Male	Louis Berndt	Stanford University

Rossia pacifica Berry

Of this widely distributed species, common on both the North American and the Japanese sides of the Pacific, the author has had opportunity to observe many specimens of different sizes. No special explanation of the structure of the statocysts is necessary, except as already stated ('24, p. 182).

Specimens examined

No.	*Locality*	*Sex*	*Collectors*	*Where deposited*
15	Off Salinas River, Monterey Bay, Calif.	Female	Fish trawl	Stanford University
16	Puget Sound, Washington	Female	Shrimp fishermen	Stanford University

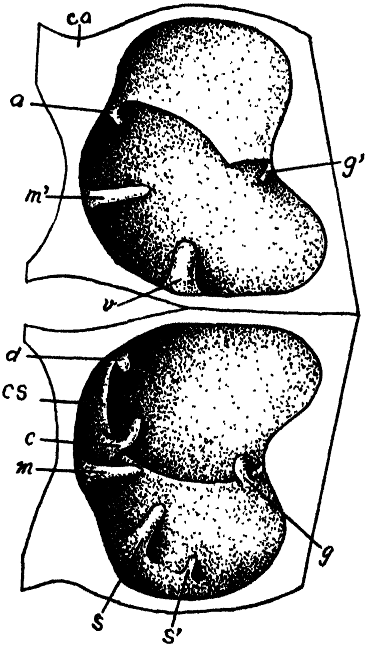

Fig. 5 Meleagroteuthis hoylei, dissections of statocyst of a specimen with a mantle length of 51 mm. (sp. no. 17). × 10. The lettering is explained in the text.

Meleagroteuthis hoylei Pfeffer (fig. 5)

In a specimen with a mantle length of 51 mm., the structure of the statocysts resembles that of Abralia and Illex; in the first region are presented five processes, *a*, *c*, *d*, *g*, and *g'*, of which the curved processes *c* and *g* are larger than *a* and *d;*

in the second region the only two processes, *m* and *m′*, are elongated; in the third region *s* is bigger than *s′*; the process *v* of the fourth region is the largest and most bulky; this species is to be placed near Abralia and Illex.

Specimen examined

No.	*Locality*	*Where deposited*
17	Albatross Station 4538	Stanford University Marine Station

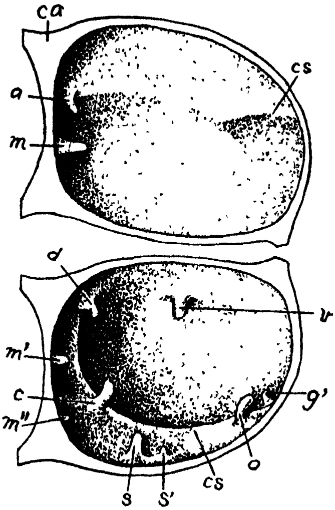

Fig. 6 Gonatus fabricii, dissections of statocyst of a specimen with a mantle length of 32 mm. (sp. no. 18). × 18. The lettering is explained in the text.

Gonatus fabricii (*Lichtenstein*) (fig. 6)

In a single specimen with a mantle length of 32 mm., five processes, *a, c, d, g,* and *g′*, of the first region are small on the whole, especially *a, d,* and *g′*, which are very small, the curved *c* and *g* processes being somewhat larger; in the second region the *m* process is somewhat larger, while *m′* is rather small, and *m″* quite rudimentary; in the third region *s* is large, and *s′* is so small that it is scarcely recognized as a rudimentary process, while the *v* process of the fourth region is rather large. The processes are eleven in number like those of Ommastrephes, but the structure of the statocyst seems to be quite primitive. Though the specimen examined was only a young animal, as stated above, the fact indicates that it is to be placed at the lowest position among the genera which possess eleven processes.

Specimen examined

No.	*Locality*	*Sex*	*Where deposited*
18	California coast, Albatross Station 4428	Juv.	Stanford University Marine Sta.

Ommastrephes hawaiiensis Berry

In this species the structure of the statocyst is, in general, the same as that of Ommastrephes sloani pacificus ('24, p. 186). In a specimen with a mantle length of 176 mm., all the processes, *a, c, d, g,* and *g'*, in the first region, are rather small in size; in the second region, the three processes, *m, m',* and *m''*, are all very large; in the third region, the *s* process is large, while *s'* is small; in the fourth region, *v* process is rather larger.

Specimen examined

No.	*Locality*	*Sex*	*Where deposited*
19	Albatross Station 4132	Juv.	Stanford University

Sthenoteuthis bartrami (LeSueur) (fig. 7)

In the statocyst of this species the processes *a, c, d,* and *g* of the first region are rather small in size, whereas *g'* only is very large; the processes *m, m',* and *m''* of the second region are also very large; *s* in the third region is larger than *s'* in the same region; *v* in the fourth region is rather large and bulky. On the whole, the structure of the statocyst resembles closely that of Ommastrephes—indicating that this animal is to be placed in the vicinity of the genus Ommastrephes.

Specimens examined

No.	*Locality*	*Sex*	*Where deposited*
20	Off Komandorski Island, Bering Sea	Female	Stanford University
21	Sunday Island, Kermadec Group	Female	Stanford University

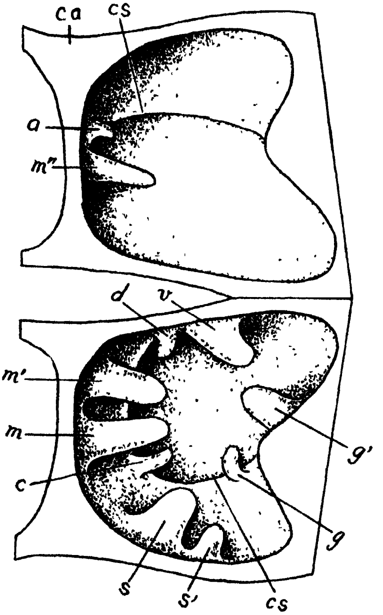

Fig. 7 Sthenoteuthis bartrami, dissections of statocyst of a specimen with a mantle length of 220 mm. (sp. no. 20). × 9. The lettering is explained in the text.

Onychoteuthis banksii (*Leach*) (fig. 8)

In this species with a mantle length of 151 mm., the processes *a, c, d,* and *g* of the first region are all rather small, but only *g'* is large and bulky; in the second region, *m, m', and m''* are all large and bulky; in the third region and the fourth, *s* and *v* are also very large, whereas *s'* is rather small. In general, the structure of the statocyst of this species is quite similar to that of the genus Moroteuthis, which has eleven processes.

Specimen examined

No.	*Locality*	*Sex*	*Collector*	*Where deposited*
22	Laysan Island	Female	Max Schlimmer	Stanford University

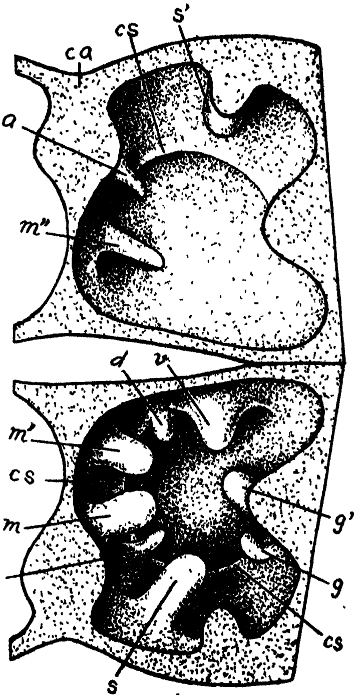

Fig. 8 Onychoteuthis banksii, dissections of statocyst of a specimen with a mantle length of 151 mm. (sp. no. 22). × 11. The lettering is explained in the text.

Lollinguncula brevis (*Blainville*) (fig. 9)

In this species the structure of statocyst is quite the same as that of the genus Loligo; in the first region of the statocyst six processes, *a*, *a'*, *c*, *d*, *g*, and *g'*, are present; of these, *a'* is extremely small, *g'* is the biggest at this region, and the others are of moderate size and about equal; in the second region three processes, *m*, *m'*, and *m''*, are very much elongated; in the third region and the fourth, *s* and *v* are bulky, while *s'* is rather small. The number of the processes is twelve in all; this fact indicates that this animal differs from others, as stated above, and is to be placed in the vicinity of the genus Loligo, which has the same number of processes.

Specimen examined

No.	*Locality*	*Sex*	*Collector*	*Where deposited*
23	Cameron, Louisiana	Female	M. H. Spaulding	Stanford University

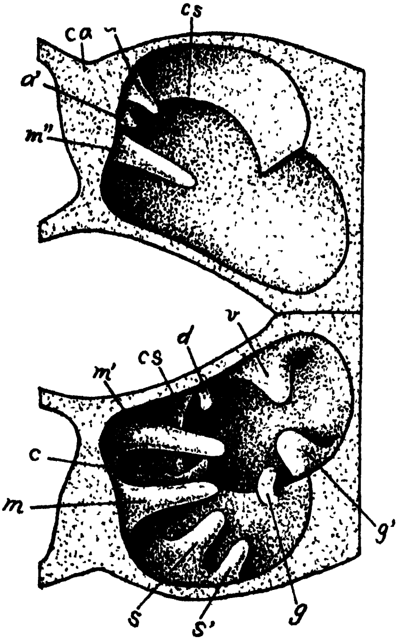

Fig. 9 Lolliguncula brevis, dissections of statocyst of a specimen with a mantle length of 43 mm. (sp. no. 23). × 20. The lettering is explained in the text.

Lollinguncula panamensis Berry

In this species the structure of the statocysts is exactly like that of Lollinguncula brevis.

Specimens examined

No.	*Locality*	*Sex*	*Collectors*	*Where deposited*
24	Panama	Female	Hopkins Expedn.	Stanford University
25	Guayaquil, Ecuador	Female	P. O. Simons	Stanford University

Loligo opalescens Berry (fig. 10)

This species is very commonly found on the North American side of the Pacific, and the structure of the statocyst is quite the same as that of the Japanese species, Loligo bleekeri, which is very near to this species. In the statocyst of this species, twelve processes, *a, a′, c, d, g, g′, m, m′ m″, s, s′*, and *v*, are observed; in the first region all the processes are generally small in size and slender, only *g′* process being large and elongated; in the second region, three processes, *m, m′*, and *m″*, are much elongated; in the third region and the fourth, both *s* and *v* are also developed and are larger than *s′*. In a specimen with a mantle length of 72 mm., the author observed, especially, only one process in the close vicinity of *s′* on the left statocyst of the animal. This is probably *s′*

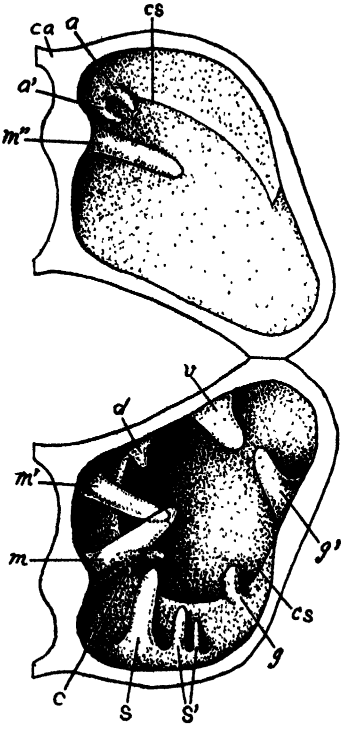

Fig. 10 Loligo opalescens, dissections of statocyst of a specimen with a mantle length of 72 mm. (sp. no. 26). × 15. The lettering is explained in the text.

divided abnormally into two processes, because in both the other specimens, with a mantle length of 118 mm. and of 135 mm., such an appended s' was not found.

Specimens examined

No.	*Locality*	*Sex*	*Collector*	*Where deposited*
26	Monterey fish market, California	Female	M. Ishikawa	Stanford University
27	Monterey fish market, California	Female	M. Ishikawa	Stanford University
28	Monterey fish market, California	Male	M. Ishikawa	Stanford University

Loligo yokoyae M. Iishikawa[2]

This is an interesting species bearing a few extraordinarily large suckers at the middle part of the right ventral arm of a male animal; this characteristic is very rare among the species belonging to the genus Loligo. But the structure of the statocyst is exactly the same as that of the above species.

Specimen examined

No.	*Locality*	*Sex*	*Collector*	*Where deposited*
29	Kagawa-Ken, Japan	Male	Y. Yokoya	Fishery Institute, Tokyo Imp. Univ.

Sepioteuthis sloani Gray (fig. 11)

In this species the structure of the statocyst as a whole is like that of the genus Loligo, the number of processes being also twelve. In the first region, five processes, a, a' c, d, and g, are moderate in size, while g' is very large; in the second region, m, m', and m'' are much developed, especially m and m' being very bulky; in the third region, s is larger than s'; in the fourth region, v is also large and bulky. The structure of the statocyst is quite the same as that of Sepioteuthis lessoniana ('24, p. 189).

Specimen examined

No.	*Locality*	*Sex*	*Collector*	*Where deposited*
30	Natal, Brazil	Male	H. Heath	Stanford University

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[2] 1926 The Proceedings of the Imperial Academy, Tokyo, Japan, no. 1, pp. 30–32; figure 1.

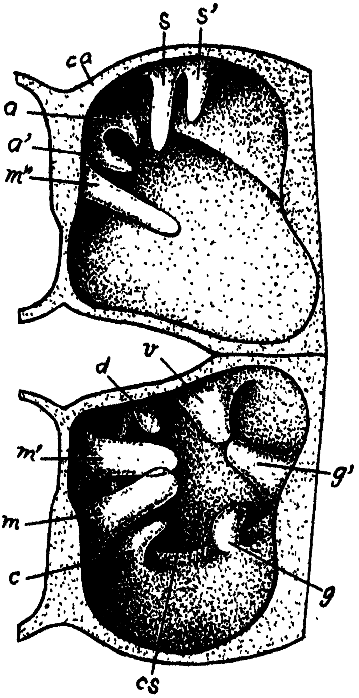

Fig. 11 Sepioteuthis sloani, dissections of statocyst of a specimen with a mantle length of 75 mm. (sp. no. 30). × 15. The lettering is explained in the text.

Sepioteuthis arctipinnis Gould

In this species the structure of the statocyst is exactly like that of Sepioteuthis sloani.

Specimens examined

No.	*Locality*	*Sex*	*Collectors*	*Where deposited*
31	Honolulu Reef, Hawaiian Islands	Male	O. P. Jenkins	Stanford University
32	Honolulu, Hawaii	Female	Branat	Stanford University

Sepia (Doratosepion) pardalis Sasaki (fig. 12)

In the statocyst of this species twelve processes are present; in the first region, six processes, *a*, *b*, *c*, *d*, *g*, and *g'*, of which *b* is to be observed only in this genus, are nearly equal to one another, while *c* and *g* are somewhat larger and strongly curved over the line of crista statica (*cs*); in the second region, three processes, *m*, *m′*, and *m″*, are well developed; in the third region, both the processes *s* and *s′* are also well developed, but *s* is bigger than *s′*; *v* in the fourth region is moderate in size. The structure of the statocyst is thus of the same type as that of Sepia (Doratosepion) tokioensis ('24, p. 193).

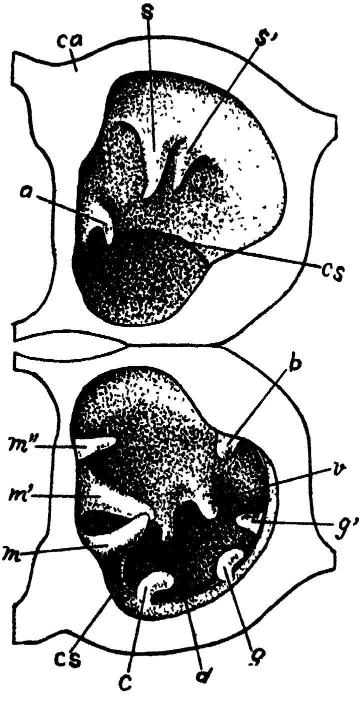

Fig. 12 Sepia (Doratosepion) pardalis, dissections of statocyst of a specimen with a mantle length of 176 mm. (sp. no. 33). × 7. The lettering is explained in the text.

Specimen examined

No.	*Locality*	*Sex*	*Collector*	*Where deposited*
33	Fuzan, Korea	Female	Y. Wakiya	Stanford Unversity

THE TABLE OF PROCESSES IN THE STATOCYST OF EXAMINED MATERIAL

In the following table the presence of each process in a species is indicated by a star.

SPECIES NO.	NAME OF SPECIES	MANTLE LENGTH, MM.	FIRST REGION							SECOND REGION			THIRD REGION		FOURTH REGION
			a	a'	b	c	d	g	g'	m	m'	m"	s	s'	v
1	Polypus bimaculatus	17	—	—	—	*	—	—	—	—	—	—	—	—	—
2	Polypus bimaculatus	30	—	—	—	*	—	—	—	—	—	—	—	—	—
3	Polypus apollyon	17	—	—	—	*	—	—	—	—	—	—	—	—	—
4	Polypus apollyon	25	—	—	—	*	—	—	—	—	—	—	—	—	—
5	Polypus macropus	30	—	—	—	*	—	—	—	—	—	—	—	—	—
6	Polypus macropus	50	—	—	—	*	—	—	—	—	—	—	—	—	—
7	Polypus oliveri	30	—	—	—	*	—	—	—	—	—	—	—	—	—
8	Polypus ornatus	26	—	—	—	*	—	—	—	—	—	—	—	—	—
9	Polypus marmoratus	40	—	—	—	*	—	—	—	—	—	—	—	—	—
10	Polypus punctatus	210	—	—	—	*	—	—	—	—	—	—	—	—	—
11	Polypus punctatus	240	—	—	—	*	—	—	—	—	—	—	—	—	—
12	Scaeurgus patagiatus	28	—	—	—	*	—	—	—	—	—	—	—	—	—
13	Euprymna scolopes	19	*	—	—	*	*	*	—	*	—	—	—	—	—
14	Euprymna scolopes	23	*	—	—	*	*	*	—	*	—	—	—	—	—
15	Rossia pacifica	19	*	—	—	*	*	*	*	*	—	—	—	—	—
16	Rossia pacifica	40	*	—	—	*	*	*	*	*	—	—	*	—	*
17	Moleagroteuthis hoylei	51	*	—	—	*	*	*	*	*	*	—	*	*	*
18	Gonatus fabricii	32	*	—	—	*	*	*	*	*	*	*	*	*	*
19	Ommastrephes hawaiiensis	108	*	—	—	*	*	*	*	*	*	*	*	*	*
20	Sthenoteuthis bartrami	220	*	—	—	*	*	*	*	*	*	*	*	*	*
21	Sthenoteuthis bartrami	280	*	—	—	*	*	*	*	*	*	*	*	*	*
22	Onychoteuthis banksii	151	*	—	—	*	*	*	*	*	*	*	*	*	*
23	Lolliguncula brevis	43	*	*	—	*	*	*	*	*	*	*	*	*	*
24	Lolliguncula panamensis	65	*	*	—	*	*	*	*	*	*	*	*	*	*
25	Lolliguncula panamensis	102	*	*	—	*	*	*	*	*	*	*	*	*	*
26	Loligo opalescens	72	*	*	—	*	*	*	*	*	*	*	*	*	*
27	Loligo opalescens	118	*	*	—	*	*	*	*	*	*	*	*	*	*
28	Loligo opalescens	135	*	*	—	*	*	*	*	*	*	*	*	*	*
29	Loligo yokoyae	. . .	*	*	—	*	*	*	*	*	*	*	*	*	*
30	Sepioteuthis sloani	75	*	*	—	*	*	*	*	*	*	*	*	*	*
31	Sepioteuthis arctipinnis	60	*	*	—	*	*	*	*	*	*	*	*	*	*
32	Sepioteuthis arctipinnis	104	*	*	—	*	*	*	*	*	*	*	*	*	*
33	Sepia (Doratosepion) pardalis	179	*	—	*	*	*	*	*	*	*	*	*	*	*

SUMMARY

1. From the structure of statocysts, in the so-called phylogenetic tree of the Cephalopoda, the genus Scaeurgus is to be placed on the same branch as Polypus.

2. Meleagroteuthis has the same number of processes as Watasenia, Enoploteuthis, Abralia, Illex, and Stigmatoteuthis, which have ten processes, but in this genus the structure of statocysts seems to be tolerably well developed. For this reason, the author places this genus as a branch between Abralia and Illex.

3. The three genera—Gonatus, Sthenoteuthis, and Onychoteuthis—which have eleven processes in a statocyst, are to be placed in the same branch as Berryteuthis,[3] Gonatopsis, Ommastrephes, Eucleoteuthis, Moroteuthis, and Symplectoteuthis, which have the same number of processes; of these, Gonatus, which has the most primitive structure of the statocyst, is to be put in the lowest position on that branch; but Sthenoteuthis, which has the same structure of the organ as Ommastrephes, is to be placed on the twig near Ommastrephes; and, lastly, Onychoteuthis has the same organ as that of Moroteuthis, which fact induces the author to set this genus in the position nearest to Moroteuthis.

4. The statocyst of Lolliguncula corresponds exactly to that of Loligo, which has twelve processes; this condition puts this genus on the same twig as Loligo.

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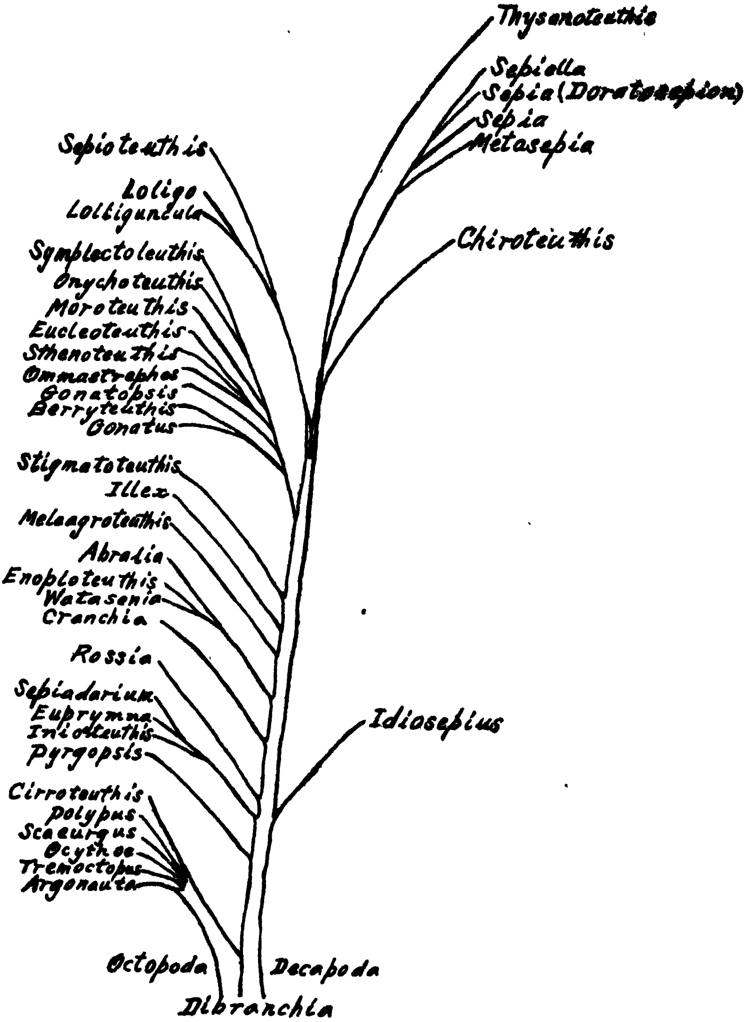
[3] In the author's previous papers this is denominated Pfferiopsis.

The foregoing may be summed up by the following so-called phylogenetic tree, which includes the previous work done by the writer, showing the supposed origin of cephalopod genera based on the structure of statocysts.



A HISTOLOGICAL STUDY OF THE COLIC CAECA IN THE BANTAM FOWL

JAMES BURDINE LOOPER AND MARGARET HAASE LOOPER
University of Mississippi

THREE TEXT FIGURES AND FIVE HELIOTYPE PLATES (EIGHT FIGURES)

AUTHORS' ABSTRACT

The caeca of fourteen bantam fowls have been studied. These fowls ranged in age from six days' incubation to three years after hatching. Between the fifth and sixth days of incubation rectal caeca arise as evaginations from the intestine at the junction of the ileum with the colon The developing caeca closely resemble histologically the intestine to which they are attached.

The caeca are essentially devoid of content until about the nineteenth day of incubation, but during the remaining days of incubation are gorged with a bluish-gray material similar to that found in the colon Thus, an early defecatory function is indicated.

In general, the proximal third of the caeca remains histologically similar to the intestine, but the distal two thirds undergoes regression The latter involves the atrophy of the epithelium and glands, accompanied by the appearance of lymphoid tissue. Much of the lymphoid tissue eventually disappears, to a large extent by atrophy and dissolution of the leukocytes. However, to some extent, lymphocytes develop into granulocytes which escape with other leukocytes into the lumina of the caeca and there disintegrate.

Lymph nodules begin to appear in the caeca about one week after the chick hatches. The leukocytes, at least in part, arise in situ from the reticular stroma. Eosinophils arise in certain areas of the tunica propria, and in the earlier stages of their development resemble large lymphocytes, in the cytoplasm of which basophilic, amphophilic, and acidophilic granules are intermingled.

CONTENTS

Introduction 585
Materials and methods 587
Observations 587
    Chicks of six to nineteen days' incubation 587
    Chicks of one to fourteen days after hatching 588
    Chicks six weeks after hatching 589
    Older bantam fowls 591
Discussion 594
    Function of caeca in the bantam 594
    Origin of lymphoid tissue in the caeca 596
Conclusions 597
Literature cited 598

INTRODUCTION

In birds there are usually two blind intestinal diverticula, the colic caeca, which have their origin at the junction of the ileum with the colon. As stated by Thompson ('25)—

In rare cases, e.g. herons, there is only one; in rarer cases, e.g. parrots, there is none. The caeca occur in all sizes, from mere vestiges, e.g. in the pigeon, to long functional tubes, as in the ducks. In the ostrich the caeca are not only very large but they contain a spiral valve. When the caeca are large enough to be of use, they serve as culs-de-sac, in which the food is delayed in its downward passage. "It is probable that in them, certain hitherto undissolved matter, as cellulose, and possibly chitin is acted upon by marsh gas, so as to extract as much nutrition as possible from the food" (Newton, '93, p. 187).

A similar diversity as regards the caeca has been shown in detail by Magnan ('11). In conclusion he states:

> Besides, we shall add that the long caeca, characteristic of a vegetarian or mixed diet, are hollow and histologically comparable to the intestine; while, the short caeca of birds which draw their food from fauna are closed and of glandular aspect.

The domestic fowl has two caeca extending forward from their ventrolateral point of origin at the junction of the small and the large intestine. These caeca, according to Kaupp ('18), average 7.61 inches in length. In the bantam the caeca average not more than 5 inches in length. However, relative to the size of the fowl, the bantam possesses longer caeca than the Barred Rock.

In the adult male the caeca average in length 2 cm. more than in the hen. This is perhaps due to the fact that the space occupied by the oviduct in the hen is left free in the male for the greater elongation of the caeca. In both the male and the female the left caecum is shorter by an average of 1.5 cm. than the corresponding caecum on the right.

The histological study of the rectal caeca in the bantam was carried out with particular reference to the origin, function, and fate of the lymphoid tissue found therein; also with reference to the function of the caeca as a whole. This work, in part, was suggested to us by Dr. H. E. Jordan, to whom we wish to express our deepest gratitude for some of the materials used and for his helpful suggestions and criticisms concerning the preparation of this paper. A part of the work was done in the Histological Laboratory of the University of Virginia.

MATERIALS AND METHODS

The materials include caeca of bantam chicks of six, nine, fourteen, fifteen, and nineteen days' incubation and caeca of postnatal bantams one day, three days, fourteen days, six weeks, six months, twelve months, fifteen months, two years, and three years of age. These tissues were fixed in Helly's formol-Zenker's fluid and stained variously with hematoxylin and eosin, eosin azure, Weigert's elastic-tissue stain, van Gieson's stain, and Foot's reticulum technique. The caeca of the younger specimens were cut longitudinally in toto. Those of the larger fowls were first cut into blocks which were designated 1, 2, 3, etc., beginning at the proximal or attached end, then sectioned longitudinally at 7 μ.

The pultaceous material, except in the younger specimens, which was found in the blind extremity of the caeca was microscopically examined in Ringer's solution.

OBSERVATIONS

Chicks of six to nineteen days' incubation

Between the fifth and sixth days of incubation the caeca arise as ventrolateral outpocketings of the intestine. The buds thus formed extend into the mesentery and remain attached to it throughout their subsequent growth and development. The growth is rapid, and early it assumes a cephalad direction along the small intestine. By the ninth day of incubation, the caeca have attained a length of 3 mm. or more; and by the fourteenth day, their blind extremities extend a short distance into the umbilical hernia. When the small intestines recede from the hernia (sixteenth to seventeenth day), the caeca are coiled backward in such manner that their distal portion points caudad. This position is the one normally found in both young and adult fowls.

Histologically, the developing caeca resemble closely the structure of the developing intestine. The primordia of villi and glands are clearly distinguishable in sections taken from the caeca of the fifteen-day chick. At this stage of development the various coats of the wall are present, viz., mucous, submucous, muscular, and outer fibroserous.

In the nineteen-day chick occasional lymphocytes may be found in the tunica propria, but granulocytes are not observed. Lymph nodules, which occur abundantly in older fowls, are not yet discernible.

The caeca of the fifteen-day chick are devoid of content, except for a trace of mucus-like material. In the nineteen-day chick, however, the lumina are gorged with a bluish-gray material, which is apparently identical with that found in the colon. Microscopically, this material contains globules of a mucinous nature, as well as occasional fragments of disintegrated yolk material. The presence of this yolk material in the digestive tract is not in accordance with Patten ('25), who states that "apparently no yolk material passes directly through the yolk-duct into the intestine." The portion of the digestive tract anterior to the attachment of the yolk stalk contains only a small amount of secretion, but the presence of considerable material in the caeca indicates that in the latter days of incubation they supplement the colon in serving a defecatory rôle.

Chicks one to fourteen days after hatching

The caeca of the one-, three-, and fourteen-day specimens measure 2.3 cm., 3.35 cm., and 5.6 cm., respectively. The one-day specimen had not been given food, and the three-day specimen had only taken crumbs of toast. The fourteen-day chick had been running free in the farm-yard since it was hatched. The caeca of the younger specimens show no evidence of bacterial infection, as was demonstrated in methylene-blue-stained smears of the caecal content. This content shows some yolk material similar to that found in the specimen of nineteen days' incubation. The fourteen-day chick, however, shows the presence of bacteria, as well as certain nematodes identified as Heterakis.

As concerns the histological structure, the caeca differ from those of earlier chicks chiefly in respect to epithelium, lymphoid tissue, and length of villi. The epithelium, which in the chick of fifteen days' incubation is several layers thick, has become essentially unilaminar and consists of tall columnar cells. Occasional goblet cells appear in the caeca of the earlier specimens. Such cells are abundant in the various stages after hatching. Lymphoid tissue is very sparse and diffuse in the one- and three-day chicks, but in the fourteen-day specimen lymph nodules occur in two regions, viz., in the tunica propria, about 2 mm. from the point of origin of each caecum (a lymphoid organ described by Ebarth), and in the tunica propria and submucosa of the blind extremities. Numerous cells are apparently rounding off from the reticulum, and mitosis in the lymphoid tissue appears rather frequently. In the one- and three-day chicks granulocytes are rare, but in the fourteen-day specimen eosinophils and basophils are relatively abundant. Villi are restricted largely to the proximal third. They have been obliterated in the distal two-thirds, presumably by distention of the caeca with collected fecal material.

Chicks six weeks after hatching

In the chick of six weeks the right caecum measures 6 cm. and the left, 5.3 cm. The histological structure of these caeca, except for certain variations to be mentioned later, is typical of those found in older forms. In the greater extent of the diverticula the wall consists of four coats, viz., mucous, submucous, muscular, and fibroserous (figs. 4, 5, and 6).

The mucosa consists of tall columnar epithelium with numerous goblet cells, a basement membrane of reticular tissue, and a tunica propria containing a large proportion of reticular tissue. The lamina muscularis mucosae is indistinct and is apparently absent in many places. The epithelium in various regions of the mucosa is infiltrated with leukocytes. The latter are also found in abundance in the lumina of the glands, as well as in the material in the blind extremity of the caeca.

The tunica propria is infiltrated with leukocytes, and in certain regions lymphocytes are apparently arising from the underlying reticulum (fig. 8). In regions where this is taking place solitary and confluent nodules are formed (fig. 6, *a, b, c*). These follicles are often crowded into the submucosa by continued development of lymphoid tissue in the tunica propria (fig. 6, *a, b, c*). Similarly, the circular muscle layer is frequently displaced in the extremity of the caeca (fig. 6). The nodules are enveloped in a rather dense reticulum which is continuous with the small amount of reticulum within the follicles. A scarcity of reticulum within the nodules is encountered in both young and old follicles. This is in accordance with our observation that the reticulum gives rise to leukocytes.

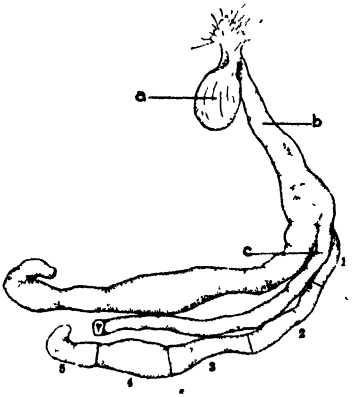


Fig. 1 Rectal caeca of bantam fowl six weeks of age. *a*, urinary bladder; *b*, colon; *c*, ileum. Figures 4, 5, and 6 are drawn from longitudinal sections of regions nos. 1, 4, and 5, respectively. × 1.

Groups of developing eosinophils are found in various regions of the tunica propria, but chiefly in the connective tissue near the submucosa (fig. 10). The granulocytes are developing apparently in situ from the lymphocytes, as close transition stages between the two are found (fig. 10, *a, b, c, d*). In the earlier stages of development there are basophilic granules intermingled with neutrophilic and acidophilic. In this stage the cells, except for the granules, closely resemble large lymphocytes. In the more mature stages of the cell the eosinophilic granules preponderate, and the nucleus assumes a polymorphic form.

In addition to the cells more commonly described as present in lymphoid tissue, the nodules contain the so-called Russell body cells and cells which closely resemble hemocytoblasts. Transition stages to both types of cells suggest origin from large lymphocytes.

The submucosa is thin relative to the other major coats. It consists chiefly of loose fibrous connective tissue, which contains relatively few elastic and reticular fibers. In regions where the lymphoid tissue is abundant in the tunica propria the submucosa is either obliterated or occupied by lymph nodules.

The muscular coat consists of cells of the smooth variety, and is subdivided into a relatively thick inner circular layer and a thin outer longitudinal layer. These layers are regular and distinct except in the blind extremity, where the circular layer is displaced by lymphoid tissue. Evidence of the displacement of this layer appears first in the chick fourteen days after hatching. The circular layer, particularly, is divided into bundles which are closely enveloped in a covering consisting chiefly of collagenous and reticular fibers. The reticular fibers extend into the bundles and encircle the individual cells. The latter condition, we believe, is one not commonly described.

The fibroserous coat is thin and compact and is enveloped by the visceral mesothelium.

Older bantam fowls

This group includes both male and female specimens. Caeca of four cocks were sectioned and studied. These fowls were four months, six months, twelve months, and fifteen months of age, respectively. The females include one specimen each of the following ages: four months, two years, and three years. Histologically, the caeca of male and female fowls are essentially identical. For that reason, ages alone will be referred to henceforth in descriptions and discussions.

The microscopic structure of the proximal one-third of the caeca in the above-mentioned fowls is quite similar to the corresponding regions in the chick six weeks of age. In

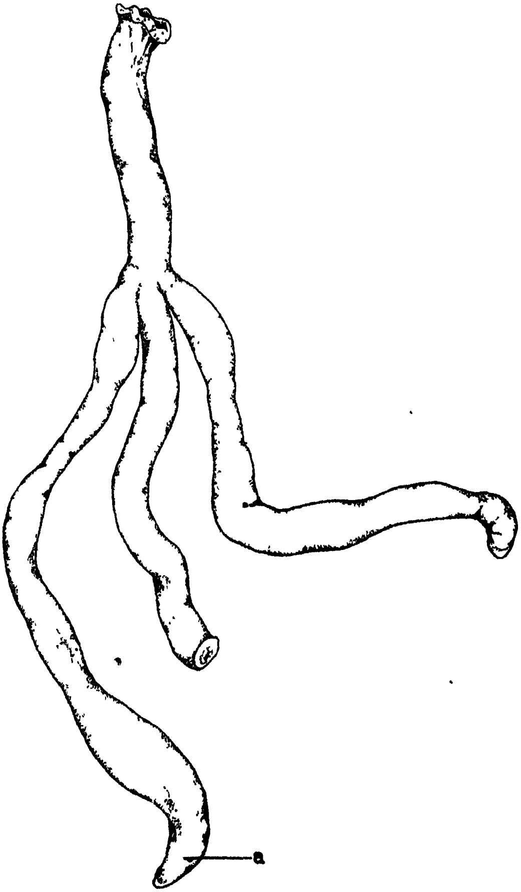


Fig. 2 Rectal caeca of bantam rooster, fifteen months of age. Figure 11 is a detail drawing from a typical degenerating lymph nodule in the blind extremity of caecum *a*. × 1.

the distal two-thirds, however, there are striking variations which involve the mucosa and the lymphoid tissue. The mucosa undergoes degenerative changes more or less progressively as the fowls become older. These changes involve the epithelium, glands, tunica propria, and lymphoid tissue.

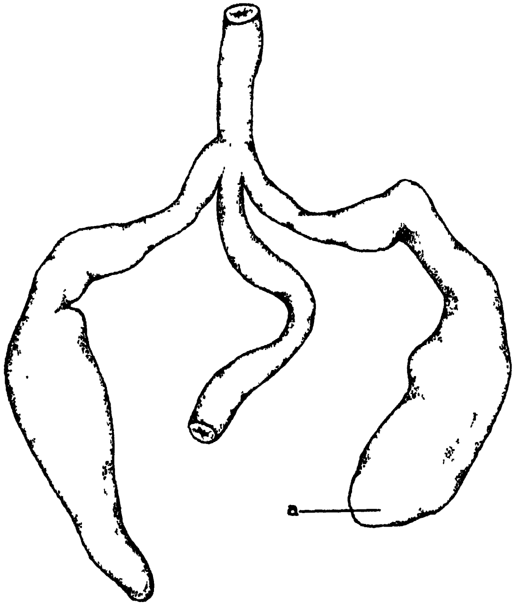


Fig. 3 Rectal caeca of bantam hen two years of age. The lower portion of the diverticula is distended with fecal-like material. Figure 7 is a drawing of a longitudinal section of blind extremity, *a*. × 1.

The epithelium varies from a tall columnar type to a more cuboidal type, and is frequently absent altogether in the blind extremity. The atrophy of the epithelium is apparently due to leukocyte infiltration subsequent to the presence in the diverticula of either bacteria or trematodes. Similarly, degeneration of the mucosal glands occurs. This is rather consistently encountered in regions where lymphoid tissue arises in abundance (fig. 6).

The tunica propria, particularly in the blind extremity of the specimen two years of age, is thin and contains relatively few lymph nodules (fig. 7, *a*). In the fowl fifteen months of age, however, the corresponding region is thick and contains much lymphoid tissue. The lymphoid tissue in both of these specimens, as well as in chicks six weeks and more of age, is undergoing degeneration (fig. 11). The atrophy is restricted chiefly to the lymph nodules, and it appears to take place gradually. Since the process is not uniform throughout the nodules, a condition results suggesting medullary spaces and cords (fig. 11).

In the blind extremity of the caeca of the fowl three years of age some of the nodules have almost completely disappeared, leaving in their place masses of sclerotic fibrous tissue. Such masses are not present in the two-year specimen, even though the lymphoid tissue has disappeared to a greater extent than in the three-year specimen. This variation is apparently due to the fact that the blind extremity of the caeca had been under pressure of distention. The latter was due to the presence of a relatively large mass of fecal-like material in the blind extremity. Thus, under the duress of pressure the fibrous tissue was unable to form to take the place of the disappearing follicles. A similar mass of material was not present in the caeca of the specimen three years of age.

DISCUSSION

Function of the caeca in the bantam

About the eighteenth day of incubation, the yolk sac and its contents are enclosed within the body cavity. From this time until hatching, considerable excreta and small amounts of disintegrating yolk material are found in the lower ileum, caeca, and colon. Since at this time the caeca are throughout histologically similar to the intestine, the presence of yolk material would suggest a possible early digestive function. However, the yolk appears only in small amounts and occasionally, so we hesitate in ascribing this digestive function to the caeca. The defecatory function, however, is quite clearly indicated, for much material similar to that in the colon occurs also in the caeca.

Mitchell ('96) showed that there is almost kaleidoscopic variety in the looping of the gut in birds, and also that these varieties are of systematic value. He made no suggestion, however, concerning the utility of either the loopings or caeca. But, as shown by Magnan ('11) and others, the caeca of carnivorous and insectivorous birds are long as compared with caeca of carnivorous birds. Magnan took this to indicate, as noted by Newton ('96), that such material as cellulose and chitin are digested, perhaps in quantity, in the longer caeca. In the bantam we have an omnivorous type of fowl, but it feeds chiefly on cereals, insects, and plants. This is particularly true of the primitive ancestor of the bantam. Therefore, the caeca of the bantam should show, according to the suggestion of Newton as well as Magnan, evidence of digesting chitin and cellulose. However, in bantam fowls of two weeks and more posthatching age we find little or no indication that such is the case. This conclusion is supported, first, by the relative absence of cellulose and chitin in the caeca; secondly, by the extensive involution of the mucosa of the caeca; and, thirdly, by the absence of cellulose-digesting organisms.

The examination[1] of the caecal content of ten different fowls, ranging in age from two weeks to three years, reveals that only small amounts of cellulose and chitin become lodged in the caeca. This is true, despite the fact that considerable amounts of both cellulose and chitin pass through the colon and are voided in the feces. Fowls which were fed on a diet rich in cellulose, such as unhusked oats, pass considerable amounts of cellulose in the feces. Caeca in fowls fed on such a diet contain no more cellulose than the caeca of fowls which were fed on food containing relatively little cellulose, e.g., unsifted corn meal. It seems possible, therefore, that caeca of fowls fed on a diet rich in cellulose should show the presence of more cellulose than the caeca of fowls fed on foods containing smaller amounts of cellulose, if the diverticula serve primarily to aid in the digestion of such.

---

[1] The presence of cellulose was determined by the identification of cell walls under the microscope; also by the use of Nowopokrowsky's iodine-zinc-chloride reagent, and Cross and Bevan's reagent. Quantitative estimates were obtained by centrifuging the material and collecting the upper portion of the mass found in the tubes. The presence of chitin was determined microscopically by the identification of fragments of insect exoskeletons and by the application of Zander's test for chitin.

The involution of the mucosa of the caeca has already been discussed in this paper under the heading, "Older bantam fowls." It might be added, however, that this degeneration involves enough of the mucosa to indicate that the caeca are not primarily concerned with a digestive function.

The examination of the caecal content of ten bantams, varying in age from two weeks to three years, failed to reveal the presence of any protozoa except an occasional cyst, taken to be that of amoeba. This material was examined fresh in Ringer's solution by means of the microscope, as well as in smears fixed in Schaudinn's fluid and stained in hematoxylin and eosin. Contents from the same caeca were cultured at 37½°C. in tubes containing strips of filter-paper and Lohnis and Lochhead's modification of McBeth's enrichment medium for cellulose-fermenting bactéria as given by Bradley and Rettger ('27). Some of these cultures were incubated as long as three months, but none showed evidence of cellulose-digesting organisms.

In view of the foregoing discussions, we are inclined to think that the caeca in bantams older than six weeks serve little or no digestive function; but, on the contrary, they are essentially vestigial appendages. However, from a study of the lymphoid tissue found in these organs, it is evident that some leukopoiesis is carried on, even in the caeca of the oldest fowl studied.

Origin of lymphoid tissue in the caeca

Lymph nodules do not appear in the caeca until a week or more after the chick hatches, but diffuse lymphoid tissue is distinguishable after the nineteenth day of incubation. The cells are at first almost wholly large and small lymphocytes. Later, however, monocytes and granulocytes appear. The earlier leukocytes apparently arise from the embryonal mesenchyma, but later the process is shifted to the reticular stroma (fig. 8). The latter observation is in accord with the observations of Lelièvre and Retterer ('10) on the third caecum (Meckel's diverticulum) of the domestic fowl. These authors, however, concluded that the glands in the blind extremity of the third caecum give rise to epithelial buds which push into the mesenchyma and develop into reticulum, then into corpuscles. Jolly ('11) describes a similar behavior on the part of the glands of the bursa of Fabricius, a cloacal diverticulum in the common fowl. However, we have not observed such behavior of the glands in the colic caeca of the bantam. On the contrary, it is evident that the glands disappear by atrophy and that the reticular tissue arises entirely from the mesenchyma.

CONCLUSIONS

The caeca of fourteen bantam fowls have been studied. These fowls range variously in age from six days' incubation to three years after hatching. The conclusions are as follows:

1. Between the fifth and six days of incubation the rectal caeca arise as evaginations from the intestine at the junction of the ileum with the colon. The developing caeca closely resemble histologically the intestine to which they are attached.

2. Until about the nineteenth day of incubation the caeca are essentially devoid of content, but during the remaining days of incubation they are gorged with a bluish-gray material similar to that found in the colon. Thus, an early defecatory function is indicated.

3. In general, the proximal third of the caeca remains histologically similar to the intestine, but the distal two-thirds undergoes regression. The latter involves the atrophy

of the epithelium and glands, accompanied by the appearance of lymphoid tissue. Much of the lymphoid tissue eventually disappears, to a large extent by atrophy and dissolution of the leukocytes. However, to some extent, lymphocytes develop into granulocytes which escape with other leukocytes into the lumina of the caeca and there disintegrate. The lymphoid tissue that disappears is replaced by fibrous tissue.

4. Lymph nodules begin to appear in the caeca about one week after the chick hatches. The leukocytes, at least in part, arise in situ from the reticular stroma.

5. Eosinophils arise in certain areas of the tunica propria. In the earlier stages of their development the eosinophils resemble large lymphocytes in the cytoplasm of which basophilic, amphophilic, and acidophilic granules are intermingled.

6. The rectal caeca in the adult bantam are essentially vestiges which apparently have little or no digestive function.

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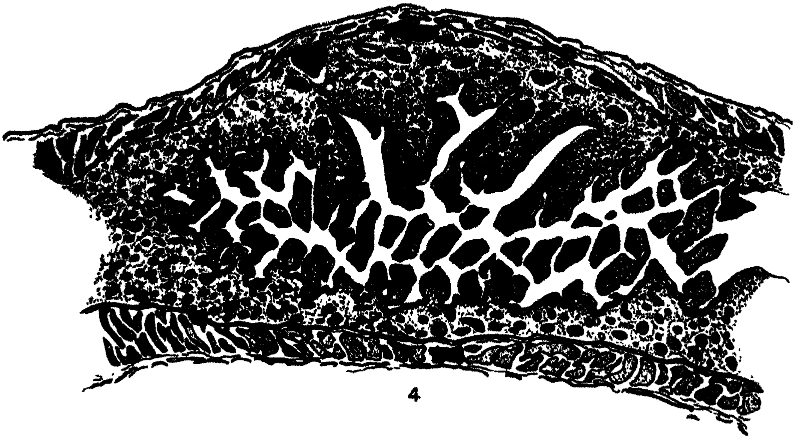
PLATES



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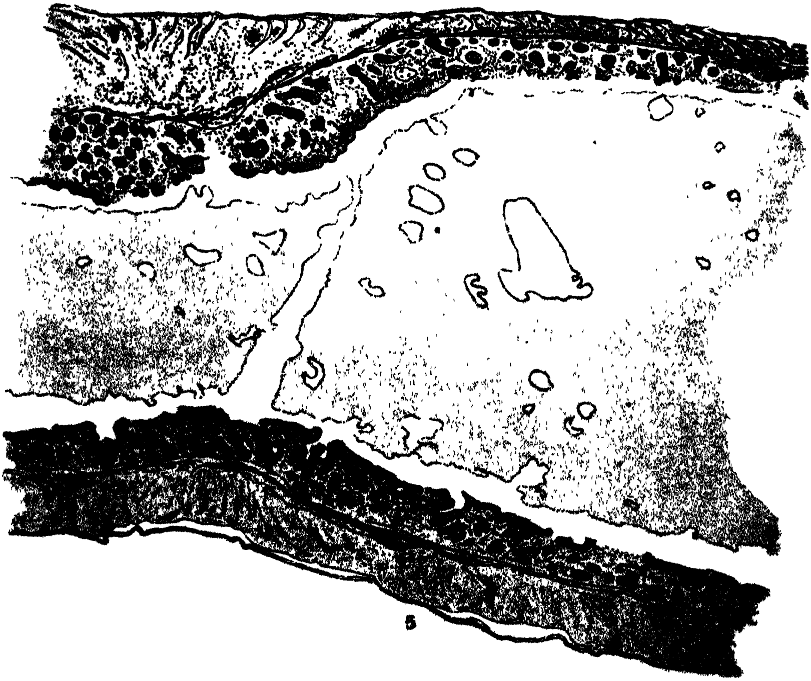

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PLATE 1

EXPLANATION OF FIGURES

4 Camera-lucida sketch of longitudinal section through region no. 1, figure 1, of bantam chick six weeks of age, showing lymph organ, described by Ebarth, at top. The lymph nodules are confined to the tunica propria and submucosa. The smaller villi are similar to intestinal villi of the chick. × 21.

5 Camera-lucida sketch of longitudinal section through region no. 4, figure 1, of bantam chick six weeks of age. Villi are absent, and the lymph nodules are located in the submucosa and between the bundles of the circular muscle. × 21.

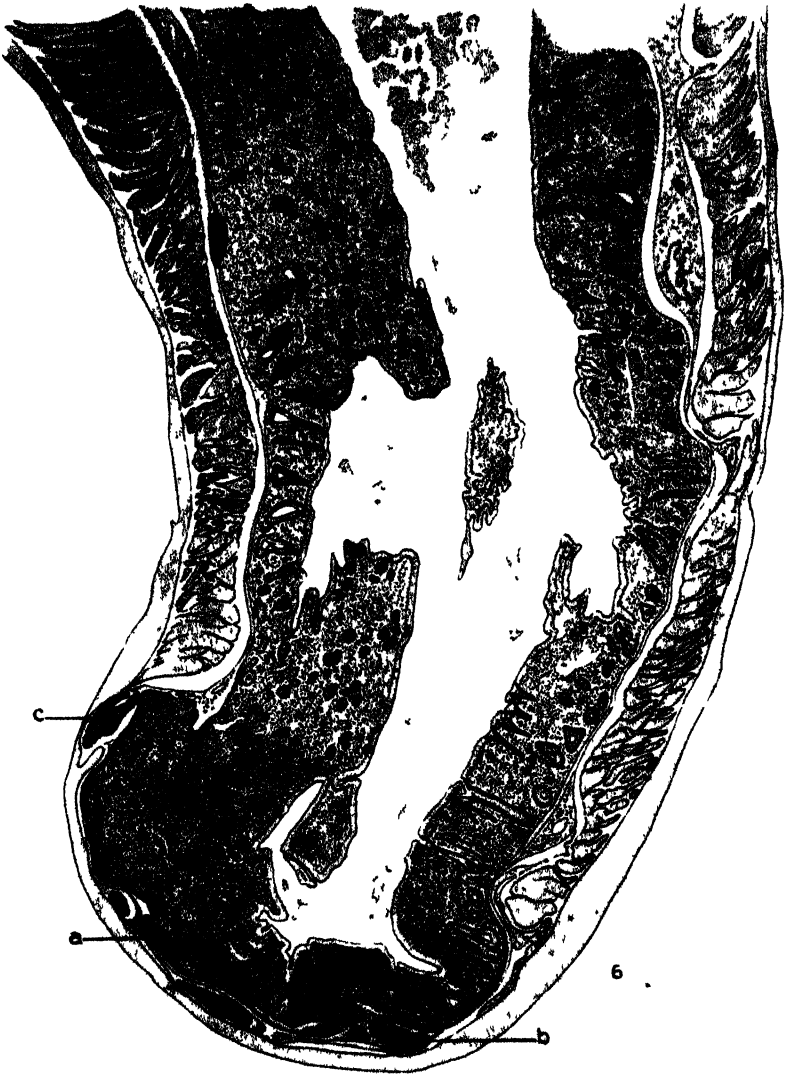
c
a
b
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PLATE 2

EXPLANATION OF FIGURE

6 Camera-lucida sketch of longitudinal section from region no. 5, figure 1, of bantam chick, six weeks of age. The submucosa and circular muscle layer have been displaced by the growth of lymphoid tissue in the blind extremity of the caecum. In the latter region some of the glands have disappeared from the mucosa. Areas *a*, *b*, and *c* show stages in the formation of lymph nodules in the mucosa. × 36.

PLATE 3

EXPLANATION OF FIGURE

7 Longitudinal section of blind extremity of caecum of bantam hen, two years of age (fig. 3, *a*). The lymphoid tissue has largely disappeared, also the epithelium and glands, leaving the mucosa thin. *a*, remnant of lymphoid tissue in the process of degeneration. × 21.

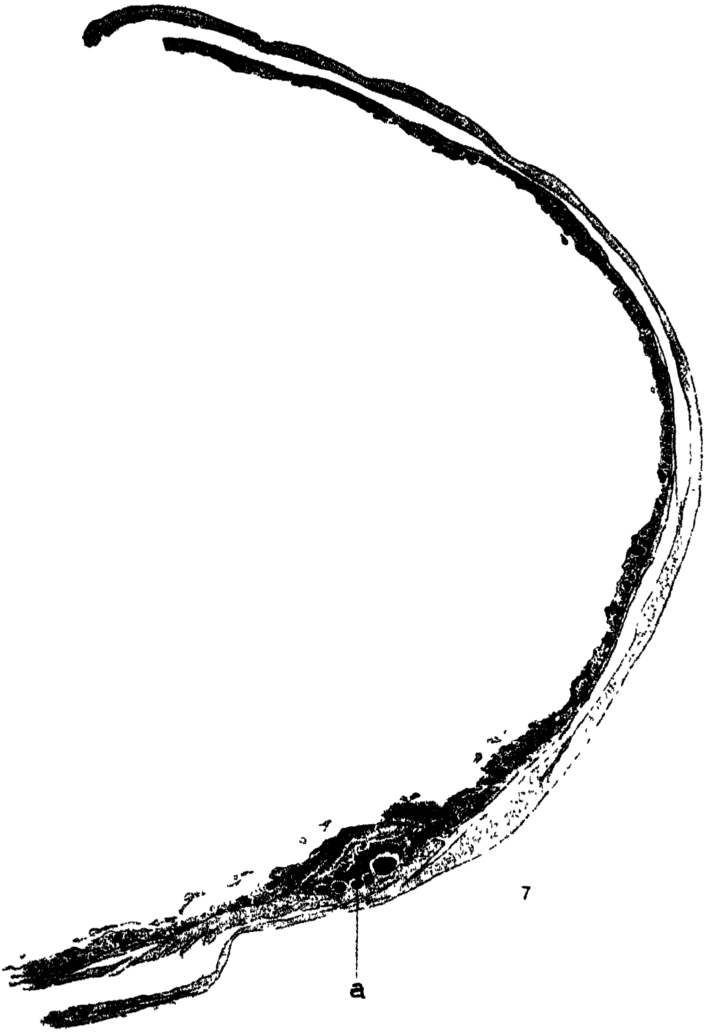

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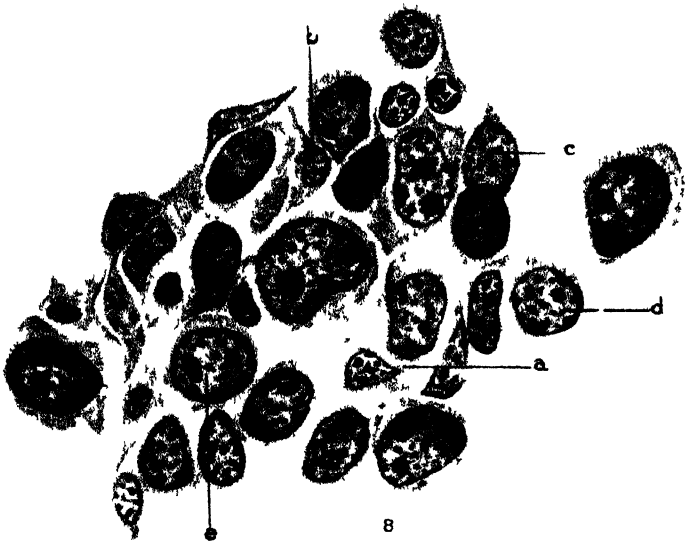
PLATE 4

EXPLANATION OF FIGURES

8 Small area from lymph nodule *b*, figure 6, showing the origin of lymphocytes from reticulum. *a*, *b*, *c*, *d*, and *e* represent successive stages in the rounding off of lymphocytes from reticulum. × 1600.

9 Small area of typical lymphoid tissue at a later stage than figure 8, showing relative absence of reticulum cells. The drawing was made from lymph nodule *c*, figure 6, of bantam chick, six weeks of age. × 1600.



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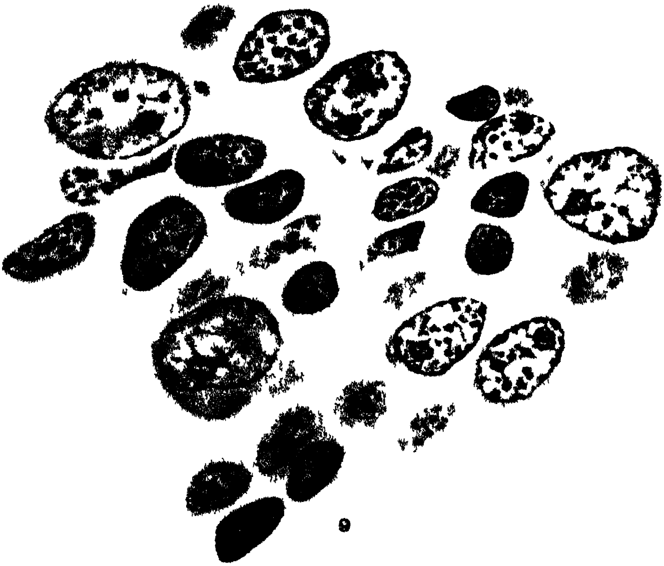

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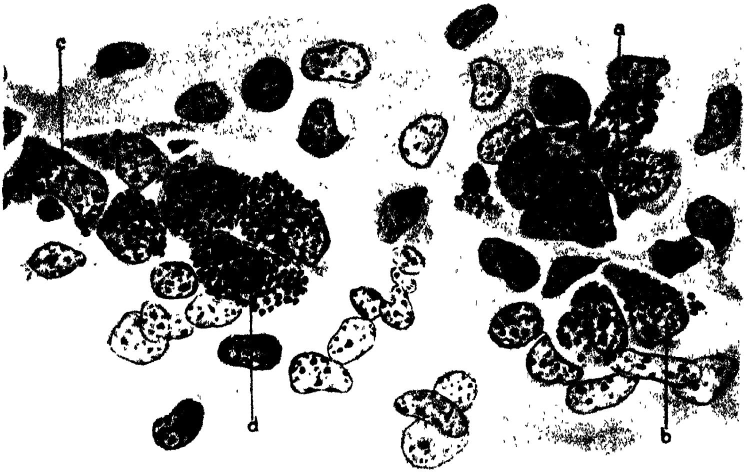
PLATE 5

EXPLANATION OF FIGURES

10 Groups of developing eosinophils in the tunica propria of a caecum of the bantam chick, six weeks of age. *a*, *b*, *c*, and *d* show successive stages in the development of eosinophils from lymphocytes. × 1600.

11 Area from degenerating lymph nodule in a caecal tip of bantam rooster, eighteen months of age, showing cords of lymphocytes and sinuses. The sinuses and the borders of the cords contain cells in various stages of disintegration. × 1600.



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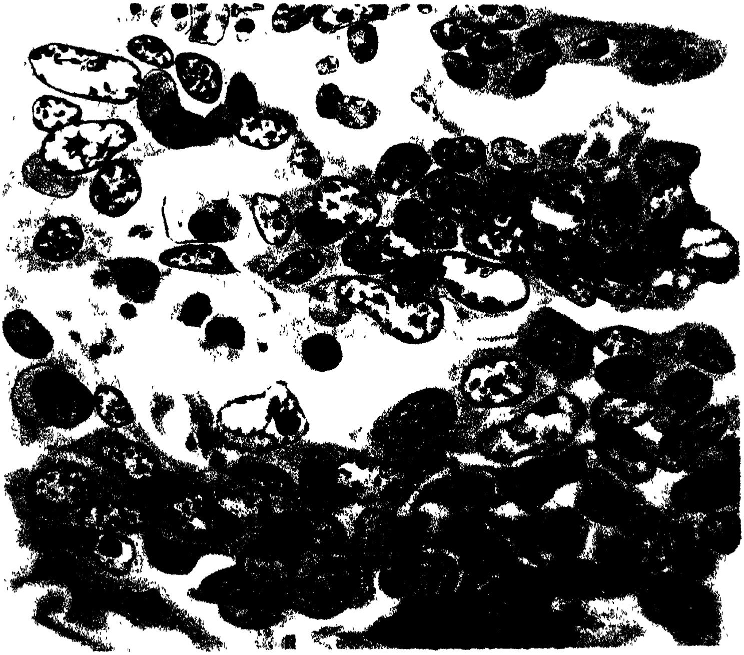

THE ORIGIN OF NEUTRAL FATS FROM THE GOLGI APPARATUS OF THE SPERMATID OF THE DOG

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ONE HELIOTYPE PLATE (SEVENTEEN FIGURES)

AUTHOR'S ABSTRACT

By the use of a new technique it was possible to demonstrate the Golgi granules about the idiosome of spermatids and in the so-called Golgi head of the mature spermatozoon. Other osmiophile granules in the cytoplasm, assumed to be Golgi granules, aggregate during metamorphosis into groups which formed neutral fat spheres, giving stains with both sudan III and scharlach R. Evidence is adduced supporting the view that the Golgi apparatus is a lipin and probably a phospholipin. The fat spheres produced are probably a result of fatty degeneration in the residual body of the spermatid They may also serve as the source of lipin in Popa's 'Lipo-gel' phenomenon.

INTRODUCTION

Since the time that Golgi discovered the so-called 'internal reticular apparatus' in 1898 until the present time there has been an abundance of research done in an effort to reveal the origin, exact nature, and function of this cell constituent. Efforts have been made to homologize the Golgi apparatus with Holmgren's trophospongium and with the vacuome of plant cells. Since the author's interest is in the function, rather than the morphology, of the Golgi apparatus and since the history of these efforts to homologize is given by several writers in the last few years (Cowdry, '23; Benoit, '26; Nath, '26; Bowen, '26; Nihoul, '26, and others), no effort will be made here to continue these comparisons, especially where there is so much confusion and so little analytical research. The purpose of this paper is to discuss the function of the Golgi apparatus and to show that in the spermatid of the dog this substance gives rise to neutral fats.

The chief function of the Golgi apparatus in sex cells, as described for the insects by Bowen ('20, '22 a, b, c, d, and '23 b) and for the guinea-pig by Gatenby and Woodger ('21), is the formation of the acroblast, which gives rise to the acrosome or perforatorium of the sperm head. That the Golgi apparatus is involved in the origin of the acrosome is now generally accepted.

Another function attributed to the Golgi apparatus is that of the elaboration of secretion products. The position of the Golgi 'network' between the nucleus and the secretory surface of gland cells has long caused the suspicion that it was involved in the production of secretion granules and liquids. The work of Nassonov ('24) might be considered as establishing this view upon a firm foundation. Bowen ('26), after a review of the literature, in this regard feels that the Golgi apparatus may "be the synthetic center wherein are built up primarily those cellular products which in the broadest sense of the term may be classified as secretions, including, therefore, some things which ordinarily pass under the name of excretions" (p. 189).

A third function, and closely akin to the second, is that of the elaboration of fats. Cajal, Bergen, Sjövall, and others regard the Golgi apparatus as being lipoidal in nature. The question as to whether yolk (especially fatty yolk) may arise from the Golgi apparatus is still a debatable one. Hirschler ('13), Cattaneo ('14), and Harvey ('25) seem to find no evidence that the Golgi apparatus in involved in vitellogenesis. Hirschler, later, however ('16), described the yolk granules as first arising from the mitochondria and then secondarily uniting with the Golgi granules. Parat and Bhattacharya ('26) concur in this view. Brambell ('24) and Rao ('27) find that one kind of yolk arises from the Golgi apparatus, while a second kind arises from the chondriome. This observation seems to be corroborated by Nath ('24, '25) and Wheeler ('24), who regard a certain kind of yolk granule as being directly developed from the Golgi material. Ludford ('21) shows that the yolk is formed in the region of the Golgi batonnettes and that there is an intimate relationship between the two which may later be disrupted, but in no case were all the batonnettes discarded by any yolk sphere. Cajal ('14) pointed out that in connective tissue the Golgi apparatus is quite unimportant, except when fat is being deposited, in which case it is fragmented. The present paper will add somewhat to the evidence that the Golgi apparatus is concerned immediately in the formation of neutral fats.

METHOD

Practically all the commonly known reagents employed for the preservation of mitochondria and Golgi apparatus, such as Kopsch's, Mann-Kopsch's, Sjövall's, Flemmings' with and without acetic, Cajal's, Da Fano's, Regaud's, Altmann's, Champy-Kull's, Benda's, and several original combinations, were used, all without sufficient success to warrant publication of observations. One has only to try these methods to convince himself of their capriciousness.

The principle involved in any fixing process is first to precipitate the protoplasmic constituents in situ, or at least to render them insoluble, and to harden them so that they may be sectioned and stained without distortion. For this reason protein precipitants are the essential factor of any good fixative. The salts of heavy metals, such as mercuric chloride; alkaloidal reagents, such as phosphotungstic, phosphomolybdic, and picric acids; strong alcohol, and many other substances are commonly employed to 'fix' protoplasm. In addition to these, acetic acid is commonly used, being well suited as a nuclear fixative.

The common difficulties encountered in using the ordinary methods of fixation are either that the protoplasm is poorly fixed or that certain inclusions, such as the lipoids, are not preserved. Fixing agents such as Bouin's fluid are excellent for preserving the natural morphology, but, unfortunately, dissolve out the lipoids. Osmic acid, which is reduced to the black oxide in the locus of lipoids, is, on the other hand, a poor fixative, at least when applied to mammalian testicular tissue. With these facts in mind, then, the author set out to find a reagent that would neither distort the protoplasm nor dissolve out the lipoids. After thorough fixation, the lipoids are to be impregnated with the osmic acid.

After more than one hundred trials with various mixtures of chemicals for different lengths of time and at different temperatures, the following combination was found to be best:

1. Formalin, strength determined according to Landolt-Börnstein tables (gravimetric) and reduced to 13⅓ per cent,	300 mls.
2. Mercuric chloride, C.P.,	7.4 grams
3. Picric acid, C.P. crystals,	1.22 grams

The following procedure was used in the successful experiments on the dog testis:

1. The animal was anaesthetized with ether, the abdominal cavity opened, a cannula inserted into the spermatic artery, physiological salt solution injected to wash out the blood, and finally the fixing agent perfused at 37°C.

2. The testis soon became yellow and hard, and was then excised and cut into thin slabs and immersed in the fixative at 37°C. for from one to twenty-four hours.

3. Washing was done in many changes of distilled water over a period of two to three hours. Lithium carbonate was added up to saturation to assist in the removal of picric acid.

4. The slabs were then immersed in 2 per cent osmic acid for from three to nine days, little difference being noted in the results. The material used for this paper was in the osmic acid for nine days.

5. The osmicated tissues were then washed and dehydrated slowly up to 85 per cent, through successive changes of ethyl alcohol in steps of 5 per cent.

6. The clearing was brought about slowly by mixing xylol in increasing strengths as dehydration was brought to completion, as follows:

87.5 per cent alcohol, 4 volumes; xylol, 1 volume; for 20 minutes
90.0 per cent alcohol, 2 volumes; xylol, 1 volume; for 20 minutes
92.5 per cent alcohol, 1 volume; xylol, 1 volume; for 20 minutes
95.0 per cent alcohol, 1 volume; xylol, 2 volumes; for 20 minutes
97.5 per cent alcohol, 1 volume; xylol, 4 volumes; for 20 minutes
100.0 per cent alcohol, 1 volume; xylol, 8 volumes; for 20 minutes
Pure xylol for about one-half hour

7. Infiltration was done slowly by adding paraffin (melting-point, 54°C.) a little at a time to the xylol. As the xylol became saturated the mixture was warmed on a thermostatically controlled hot-plate, and more paraffin was added. Finally, the tissues were moved into pure paraffin, and after a half hour or more at a temperature just above the melting-point they were changed again into fresh paraffin. Two or three changes may be necessary to insure the complete removal of the xylol, which, if present, would make fine sectioning difficult.

8. The tissues were then embedded in pure paraffin.

9. Sections were cut at 5 μ, although by the above technique tissues could be cut down to 1 μ by properly controlling the temperature while sectioning.

10. The ribbons were fixed on slides in the usual way. After drying, the paraffin was dissolved in xylol and a cover-slip was mounted in balsam. It was unnecessary to tone or to stain the tissues, as the cytoplasm assumed a light gray with black inclusions, while the nucleus remained paler. Acid fuchsin saturated in anilin water was used to some extent, however, to stain chromatin structures.

The above fixatives were selected because they were known to be used extensively as fixing agents. Bichloride of mercury not only is a good protein precipitant, but also forms an insoluble compound with lecithin. This may be the reason that it is used in the Mann-Kopsch mixture. Formalin was used on general principles, its efficacy as a tissue preservative being well established by long experience. Experiments by the author showed, however, that formalin used alone was inferior to the mixture above. The picric acid was used experimentally, since there was little evidence that it was likely to dissolve out the lipoids and since it is well known for its rapid penetration. There is, however, the possibility of picric acid dissolving out the lecithin that may be present (Lüdecke, '05), but this would occur slowly and the length of time of exposure can be controlled.

The author wishes to draw attention to the fact that the above technique has not always given results that are satisfactory, but that results are obtainable that cannot be secured by other techniques.

OBSERVATIONS

Although it is not the purpose in this paper to review any work on the spermatogenesis of the dog (Malone, '18), it will be convenient to treat of certain aspects of mammalian spermateliosis, in order to correlate the behavior of the lipoid constituents of the spermatids with the chronology of cellular events. To accomplish this, it has seemed best to consider the metamorphosis of the spermatid as being divided into three periods, which are discussed as follows:

Period I

This period may be said to be initiated with the 'birth' of the spermatid following the second maturation division. The nucleus at first shows vestiges of the telophase chromosomes and also the karyosome, but only for a very brief space of time, as the contents soon become homogeneous. The cytoplasm is relatively clear at this stage and contains very few granules which are blackened by osmic acid, indicating the absence of any extensive amount of reducing substances, such as unsaturated lipoids. The idiosome is at first slightly removed from the nucleus and is surrounded by a number of small osmiophile granules (fig. 1). These granules are undoubtedly the homologue of the Golgi apparatus described for the guinea-pig by Gatenby and Woodger ('21), but at no time do they assume a crescentic shape like those of Cavia. Soon the idiosome comes to lie against the nucleus (fig. 2), but very quickly is repelled again by the proacrosomal vacuole which arises between the two (fig. 4). This vacuole is undoubtedly the homologue of the archoplasmic vacuole described for the guinea-pig by Gatenby and Woodger ('21). It is at first spherical and, upon growing to its full size, seems to exert a pressure upon the nucleus, since the latter is pressed in at this surface. In the center of the concavity thus produced upon the anterior end of the nucleus there is formed a granule, which is destined to be the acrosome of the adult sperm.

Period II

During the early part of this period the shape of the proacrosomic vacuole is altered by becoming flattened and subsequently concave on the nuclear surface, while the nucleus returns almost to its original contour (figs. 6 and 7). There is also a great increase in the number of osmiophile granules in the cytoplasm, developing apparently de novo, as there are no indications of dictyokinesis or fragmentation of particles. The important thing to note during this period is the aggregation of these granules to form larger spheres (figs. 7 and 8). The nature and significance of these granules and spheres will be discussed elsewhere in this paper. This period closes with the recession of the residual body of cytoplasm to the caudal part of the cell and with the outgrowth of a filament (fig. 10).

Period III

This period is marked by the shrinking and elongating of the nucleus to form the sperm head, by the discarding of the residual cytoplasm, and by the subsequent fate of the sperm. A thing of especial note during the latter part of period II and all of period III is the relative paucity of the small osmiophile granules—a fact correlated with their function of forming the larger spheres. Toward the end of this period, the sperm cell discards its residual cytoplasm, not by casting it off caudally, but by backing out of it (fig. 14), as has been described for the opossum by Duesberg ('20). When the mature spermatozoon has been formed, there is a protoplasmic sphere impregnated with osmiophile granules located just below the head and about the middle piece. This sphere has been called the Golgi bead by Gatenby and Woodger ('21), who show that the structure is quite common in the higher animals. There is no evidence, however, that the Golgi apparatus about the idiosome of the dog divides, one part becoming localized as the Golgi bead and the other being discarded in the residual cytoplasm, as in the guinea-pig.

DISCUSSION

The reasons for considering the small osmiophile granules of the cytoplasm as the same as what is called the Golgi apparatus by others are threefold, namely: they are about the same size and shape (spherical) as are the Golgi granules about both the idiosome and the Golgi bead; they appear when the idiosome and Golgi-bead granules appear; and they do not appear when techniques are employed that do not show the Golgi granules of these two structures—at least if granules are present such as those in mitochondrial preparations (fig. 16), they do not show aggregations. On the other hand, the granules are not considered to be mitochondrial, for certain cells allowed to remain in the fixative for twenty-four hours do not show the Golgi elements shown above, but do have granules that arrange themselves along the middle piece precisely as do the mitochondria (figs. 16 and 17).

The question now becomes one of identifications of the fine granules and the larger spheres. That they are both lipoidal is at once suspected by the fact that they both reduce osmic acid. That the fine granules are an unsaturated lipoid is probable from the fact that they cause a greater reduction of the osmic acid, i.e., the blackening is more intense. The larger spheres give a dull gray coloring, which is easily dissolved out in turpentine. This is characteristic of the neutral fats. To test this further, fresh smears and frozen sections were made and stained in sudan III and in Herxheimer's scharlach R. All preparations gave a decided coloring of the spheres. This is proof that they are composed of neutral fats.

The exact chemical nature of the smaller granules, however, is more difficult of solution, and a probable answer to the question of their character may at the present only be obtained by inference. To isolate the granules and test them chemically is beyond the technique of modern cytology or biochemistry. If a number of small granules give rise to a neutral fat, there is apparently either an oxidation or saturation of triglyceride whose fatty-acid radicals are unsaturated, or a transformation of a phospholipin into a neutral fat. That the latter view is more nearly correct may be so regarded from the following facts:

a. The phospholipins, and especially lecithin in combination with cholesterol, are universal cell constituents; they are the so-called 'éléments constants' of the French workers; i.e., in starvation they are not used up as are the fat-depots.

b. The phospholipins are to some extent miscible in aqueous solutions. They thus serve as a form in which the unavailable fat may be used by the protoplasm. Phospholipins are capable of existing in the colloidal form.

c. The fats of milk are probably derived from the phospholipins of the blood of the cow. Meigs, Blatherwick, and Cary ('19) give evidence to show that most, if not all, of the milk fat is formed from phospholipins of the blood. Others have found that a phosphorus-low diet impaired the milk-fat production (Bloor, '25, p. 280) as evidence that the phospholipins act as intermediary stages in fat-metabolism.

d. The phospholipins are chemically more reactive than the fats, being more readily oxidized and more easily hydrolyzed than are the simple triglycerides. This fact, together with those above, is offered by Bloor ('25, p. 280) as evidence that the phospholipins act as intermediary stages in fat-metabolism.

e. When in solution, lecithin, phrenosin, and other phospholipins are able to exhibit myelin forms, due to their low surface tensions. Streamers and bands extend from the surface under certain conditions, giving figures that might easily be identical with those often described for the Golgi apparatus of gland cells, etc. (Leathes, '23 and '25; Rosenheim, '14).

f. Lastly, it is not without significance that Walker and Allen ('27) found that colloidal mixtures of lecithin and kephalin, when fixed upon a slide and treated for Golgi apparatus, gave structures exactly like the cellular Golgi elements. A trace of acetic acid in the fixative, however, did not permit of the same results. It is a well-known fact that fixing agents containing acetic acid do not preserve the Golgi apparatus. Acetic acid is an excellent solvent for lecithin (Maclean and Maclean, '27, p. 21).

What the function of the spheres of neutral fat, which have been formed from the fine osmiophile granules and are destined to be discarded with the residual body into the seminal fluid, may be is a matter of conjecture. They may represent merely a fatty degeneration. According to Bloor ('25, p. 277), "The apparent increase of fat in degenerating tissues is due to a setting free of fatty material which in normal tissues is 'built in' in such a way as to be invisible, unstainable by histologic methods and not extractable with ether." That they may have a greater significance than that mentioned above is suggested by researches of Popa ('27), who discovered that a mixture of spermatozoa and follicular fluid produced a gel, named by him the 'lipo-gel.' He also discovered that the essential substance in the seminal fluid was lipin material, and that lipins from almost any other organ of the body would give identical results. The formation of the lipo-gel may have a great significance, since, as Popa points out (p. 237), the "spermatozoa furnish a very good medium for the growth of bacteria." He continued that "The agglutination of the spermatozoa by the follicular fluid would hold them to be phagocytized more readily, thus protecting the peritoneum from possible infection."

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PLATE 1

EXPLANATION OF FIGURES

Figures 1 to 15 are approximately 2750 diameters. Figures 16 and 17 are about 3500 diameters. All are camera-lucida drawings, the microscope being fitted with fluorite oil-immersion objectives and compensating oculars. Drawings reduced one-sixth in reproduction.

1 to 5 Early stages of deveopment of the spermatid, showing the idiosome with its osmiophile granules and, later, the proacrosomic vacuole.

6 to 8 Spermatids, showing increase of number of osmiophile granules and their aggregation to form fat-spheres.

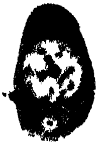
9 to 13 Later stages in development of spermatid, in which the cytoplasm recedes caudally and the nucleus becomes elongated to become the typical sperm head.

14 Sperm nearly mature, withdrawing from the residual mass of cytoplasm.

15 Mature spermatozoon, exhibiting the middle-piece 'Golgi bead.'

16 Nearly mature sperm, fixed so as to show the mitochondria.

17 A portion of a seminiferous tubule, fixed also to show the mitochondria. Besides the Sertoli cell and its filiform mitochondria, there are one primary spermatocyte and two spermatids visible.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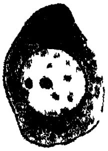
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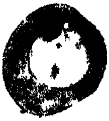
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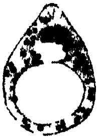
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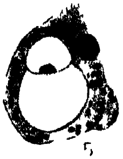
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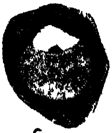
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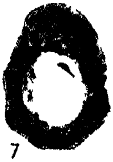
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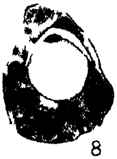
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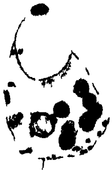
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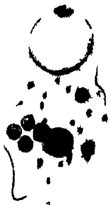
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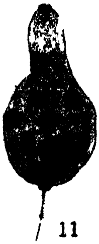
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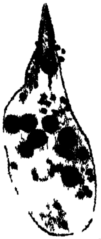
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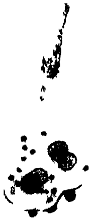
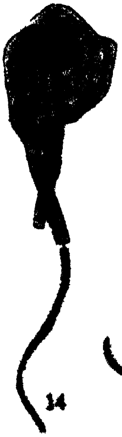
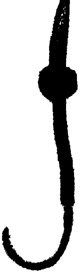
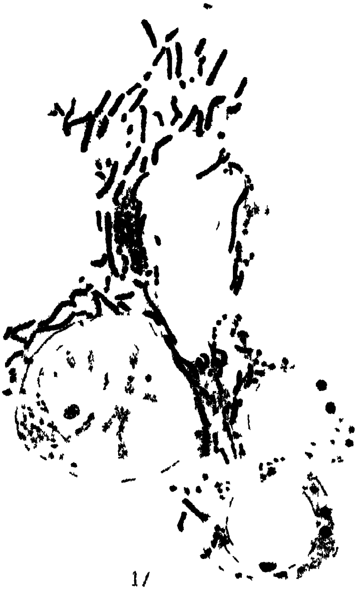

13


14


15

16


17

INDEX

ADRENAL gland of the albino rat (Mus norvegicus albinus). The influence of thyroid deficiency on the structure and epinephrin content of the 45

ALLEN, EZRA, AND A. M. BANTA. Growth and maturation in the parthenogenetic and sexual eggs of Moina macrocopa 123

Amoeba proteus. The effects of the substratum, divalent and monovalent cations on locomotion in 371

Anura. A histological study of the parathyroids in the 1

Arterial system of the lizard Uromastix hardwickii Gray. On the 281

BANTA, A. M. See Ezra Allen 123

BELL, A. W. The origin of neutral fats from the Golgi apparatus of the spermatid of the dog 611

BHATIA, M. L. On the arterial system of the lizard Uromastix hardwickii Gray 281

BLOUNT, RAYMOND F. Seasonal cycles of the interstitial cells in the testis of the horned toad (Phrynosoma solare). Seasonal variations in the number and morphology of the interstitial cells and the volume of the interstitial tissue 317

CASTLE, W. E., AND P. W. GREGORY The embryological basis of size inheritance in the rabbit 81

Cations on locomotion in Amoeba proteus. The effects of the substratum, divalent and monovalent 371

Cephalopod genera. On the statocyst of the American 563

CHENG, TSO-HSIN. Intersexuality in Rana cantabrigensis 345

Colic caeca in the bantam fowl. A histological study of the 585

CURRY, LALIAH F. A cytological study of the proximal and distal tubules of the mesonephros of Necturus maculosus. (Under normal and experimental conditions.) 173

DIACRISIA virginica Fabr. (Lepidoptera). Some phases in the embryonic development of 493

Diplopods. I. The development of the external sexual structures of Parajulus impressus Say. Studies of parajulid 153

Dog. The origin of neutral fats from the Golgi apparatus of the spermatid of the 611

Dyes and their relation to mitochondria and Golgi apparatus in the flagellate Peranema trichophorum. Reaction of certain cytoplasmic inclusions to vital 105

EGGS of Moina macrocopa. Growth and maturation in the parthenogenetic and sexual 123

Epididymis. II. The importance of an aging process in sperm for the length of the period during which fertilising capacity is retained by sperm isolated in the epididymis of the guinea-pig. A study of the function of the 475

FATS from the Golgi apparatus of the spermatid of the dog. The origin of neutral 611

Fishes. The parietal fossa and related structures in the plagiostome 543

Flagellate Peranema trichophorum. Reaction of certain cytoplasmic inclusions to vital dyes and their relation to mitochondria and Golgi apparatus in the 105

Fowl. A histological study of the colic caeca in the bantam 585

GERM cells in the rat. Maturation of the male 433

GEROULD, JOHN H. Periodic reversal of heart action in the silkworm moth and pupa 385

Golgi apparatus in the flagellate Peranema trichophorum Reaction of certain cytoplasmic inclusions to vital dyes and their relation to mitochondria and 105

Golgi apparatus of the spermatid of the dog. The origin of neutral fats from the 611

GREGORY, P. W. See W. E. Castle. 81

Guinea-pig. A study of the function of the epididymis. II. The importance of an aging process in sperm for the length of the period during which fertilizing capacity is retained by sperm isolated in the epididymis of the 475

HALL, RICHARD P. Reaction of certain cytoplasmic inclusions to vital dyes and their relation to mitochondria and Golgi apparatus in the flagellate Peranema trichophorum 105

Heart action in the silkworm moth and pupa. Periodic reversal of 385

HEFNER, R A. Studies of parajulid diplopods. I. The development of the external sexual structures of Parajulus impressus Say 153

HOPKINS, DWIGHT L. The effects of the substratum, divalent and monovalent cations on locomotion in Amoeba proteus 371

Horned toad (Phrynosoma solare). Seasonal variations in the number and morphology of the interstitial cells and the volume of the interstitial tissue. Seasonal cycles of the interstitial cells in the testis of the 317

INTERSEXUALITY in Rana cantabrigensis 345

Interstitial cells in the testis of the horned toad (Phrynosoma solare). Seasonal variations in the number and morphology of the interstitial cells and the volume of the interstitial tissue. Seasonal cycles of the 317

ISHIKAWA, MASASHI. On the statocyst of the American cephalopod genera 563

JOHANNSEN, O. A. Some phases in the embryonic development of Diacrisia virginica Fabr. (Lepidoptera) 493

(LEPIDOPTERA). Some phases in the embryonic development of Diacrisia virginica Fabr. 493

Lizard Uromastix hardwickii Gray. On the arterial system of the 281

Locomotion in Amoeba proteus. The effects of the substratum, divalent and monovalent cations on . . 371
LOOPER, JAMES BURDINE, AND MARGARET HAASE LOOPER. A histological study of the colic caeca in the bantam fowl 585
LOOPER, MARGARET HAASE. See James Burdine Looper 585

MATURATION of the male germ cells in the rat 433
Mesonephros of Necturus maculosus. (Under normal and experimental conditions.) A cytological study of the proximal and distal tubules of the 173
Mitochondria and Golgi apparatus in the flagellate Peranema trichophorum. Reaction of certain cytoplasmic inclusions to vital dyes and their relation to 105
MOHAN, PIARE. See Vishwa Nath 253
Moina macrocopa. Growth and maturation in the parthenogenetic and sexual eggs of 123
(Mus norvegicus albinus). The influence of thyroid deficiency on the structure and epinephrin content of the adrenal gland of the albino rat 45

NATH, VISHWA, AND PIARE MOHAN. Studies in the origin of yolk. IV. Oogenesis of Periplaneta americana . . 253
Necturus maculosus. (Under normal and experimental conditions.) A cytological study of the proximal and distal tubules of the mesonephros of 173
NORRIS, H. W. The parietal fossa and related structures in the plagiostome fishes . 543

OOGENESIS of Periplaneta americana. Studies in the origin of yolk. IV. 253
Ovarian chromosome cycle in a mixed rat strain. The 445

PARAJULUS impressus Say. Studies of parajulid diplopods. I. The development of the external sexual structures of 153
Parathyroids in the Anura. A histological study of the . . 1
Parietal fossa and related structures in the plagiostome fishes. The . 543
Peranema trichophorum. Reaction of certain cytoplasmic inclusions to vital dyes and their relation to mitochondria and Golgi apparatus in the flagellate 105
Periplaneta americana. Studies in the origin of yolk. IV. Oogenesis of 253
(Phrynosoma solare). Seasonal variations in the number and morphology of the interstitial cells and the volume of the interstitial tissue. Seasonal cycles of the interstitial cells in the testis of the horned toad 317

RABBIT. The embryological basis of size inheritance in the . . 81
Rana cantabrigensis. Intersexuality in 345
Rat. Maturation of the male germ cells in the 433
Rat (Mus norvegicus albinus). The influence of thyroid deficiency on the structure and epinephrin content of the adrenal gland of the albino 45
Rat strain. The ovarian chromosome cycle in a mixed 445

SEXUAL structures of Parajulus impressus Say. Studies of parajulid diplopods. I. The development of the external 153
Silkworm moth and pupa. Periodic reversal of heart action in the . 385
Size inheritance in the rabbit. The embryological basis of 81
Sperm for the length of the period during which fertilizing capacity is retained by sperm isolated in the epididymis of the guinea-pig. A study of the function of the epididymis. II. The importance of an aging process in 475
Spermatid of the dog. The origin of neutral fats from the Golgi apparatus of the 611
Statocyst of the American cephalopod genera. On the 563
Substratum, divalent and monovalent cations on locomotion in Amoeba proteus. The effects of the . . . 371
SUN, TSON PEN. The influence of thyroid deficiency on the structure and epinephrin content of the adrenal gland of the albino rat (Mus norvegicus albinus) 45
SWEZY, OLIVE. Maturation of the male germ cells in the rat . 433
SWEZY, OLIVE. The ovarian chromosome cycle in a mixed rat strain . 445

TESTIS of the horned toad (Phrynosoma solare). Seasonal variations in the number and morphology of the interstitial cells and the volume of the interstitial tissue. Seasonal cycles of the interstitial cells in the 317
Thyroid deficiency on the structure and epinephrin content of the adrenal gland of the albino rat (Mus norvegicus albinus). The influence of 45
Tubules of the mesonephros of Necturus maculosus. (Under normal and experimental conditions.) A cytological study of the proximal and distal 173

UROMASTIX hardwickii Gray. On the arterial system of the lizard. 281

WAGGENER, ROY A. A histological study of the parathyroids in the Anura 1

YOLK. IV. Oogenesis of Periplaneta americana. Studies in the origin of 253
YOUNG, WILLIAM C. A study of the function of the epididymis. II. The importance of an aging process in sperm for the length of the period during which fertilizing capacity is retained by sperm isolated in the epididymis of the guinea-pig 475

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